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ELECTRICAL CONTROL OF RHIZOID FORMATION IN THE RED ALGA, GRIFFITHSIA BORNETIANA*

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I

INTRODUCTION

The significance of electricity in basic vital activities has been the subject of much speculation ever since potential differences were detected in living organisms. Among other things, fundamental guiding powers in morphogenesis have been ascribed to electrical forces. This paper is concerned with the use of electric current in the analysis of organic polarity. Experimental work of this kind also shows promise for a proper evaluation of electrical energy in life phenomena.

Investigation along this line may be traced to the observation of Elfving (1882) who found that the radicles of most seedlings will turn and grow toward the cathode. Lowenherz (1908), early in the present century, contributed the next significant forward step when he pointed out that the orientation of seeds germinating in an electrical field affected the rate of growth. These two workers demonstrated the polar action of the current and polarity in the organism.

Mathews (1903) measured potential differences in the hydroid *Tubularia* and found the polyp surface to be electronegative to the stolon surface. On the basis of this and other work he suggested that "the physiological polarity of the embryo or adult is due in a measure at least to the electrical differences or currents set up by an unequal degree of activity in the protoplasm at different regions. These currents traverse the surrounding protoplasm or cells and like any constant current applied from outside polarize the protoplasm or cells in a definite way,

1

causing alterations in their metabolism, and in the distribution of the cell contents   ”

In 1920, in a brief but interesting note Ingvar states that weak galvanic currents of the same order of magnitude as bioelectric currents normally reported for embryos, will affect the nature and direction of outgrowth of chick nerve cells *in vitro*   Here for the first time there is experimental evidence for the correlation claimed between electrical forces and normal formative processes   The most extensive work in investigating this phenomenon has been done with hydroids Lund published a series of papers (1921–1924) showing that cathodal inhibition of polyp production by *Obelia* internodes takes place in a range of 53 to 130 microamperes per sq mm of cross-section of sea water   It also appeared that the more apical internodes which are electrically more positive have a greater power of resistance to the inhibition   Lund suggested that inhibition of regeneration is due to reversal of the inherent bioelectric potential   In a further analysis of this relationship Barth found that electrical reversal of physiological and structural polarity, in *Tubularia*, is indeed accompanied by a change in direction of the bioelectric current   But Barth (1934) points out, in a tabulation of the results with various species of hydroids used in his own and in Lund's work, that inhibition occurs in some forms at the anode and in some at the cathode, also that the sign of the inherent current at the apical end of the hydroid varies with different forms   It was also found that in *Tubularia* reversal of the pole at which inhibition occurs appears with high currents   Thus the simple explanation offered by Mathews, and hitherto accepted, becomes untenable   The possibility is even introduced that the bioelectric current is perhaps more of a symptom than a cause of physiological dominance

It appears that plants, particularly because of their simpler symmetry, would be likely to give a clearer insight into the nature of the action of a bipolar force such as electricity   Besides, electrical modification of polarity in differentiated plant thalli[1] had never been demonstrated and before any very general conclusions are drawn, or any very fundamental significance attached to electrical action, a broader experimental basis is desirable   The experimental part of this paper is offered as a step in this direction

II

Material and Methods

Explorative experiments were performed upon several likely species of algae and liverworts   I wish to report in this paper the results obtained with the red

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[1] Lund (1923) has shown in the *Fucus* egg that the first cleavage plane becomes oriented in relation to the electric field

alga, *Griffithsia bornetiana* Farlow, as this form responded most clearly and completely to electrical stimulation. The work was done at the Marine Biological Laboratory at Woods Hole during the summers of 1932 and 1933.

Griffithsia thalli usually develop from spores. After several nuclear divisions the spore cleaves once to form the rhizoid and then again to form the shoot (Fig 1) Under laboratory conditions this occurs within a day after the spores are shed and at this time the normal polarity of the plant is already established. The mature thallus consists of branched chains of large club shaped multinucleate cells (Fig 2) which vary in size from about 0 06 mm in diameter at the apex to 0 65 by 2 5 mm at the base. The anterior posterior axis is clearly marked by the distinctive club shape with wider portion at the apex. Each cell contains from 12 to 4,000 nuclei (Lewis 1909) depending upon its age. The older basal cells produce long hair like rhizoids which serve to attach the plant (Fig 3). The cell possesses a cellulose wall which encloses a thin cytoplasmic layer containing the nuclei, chromatophores, and other formed bodies. The chromatophores are most numerous. They are 50 to 100 times as plentiful as the nuclei. The bulk of the cell consists of the large central vacuole. Pores bordered by a thickened layer of cytoplasm, lead from one cell to another. The diameter of the pores is about 10 micra three times that of nuclei or chromatophores. Rhizoids originate as an accumulation of protoplasm and a concentration of pigment which pushes out at the head of a tubular prolongation of the vacuole of the mother cell. New shoots, on the other hand, are at once separated from the parent cell by cross walls.

The material used in the experiments was obtained in the tide wash at Nobska Point. It is first found about the middle of July and becomes scarce again toward the middle of August. As the season draws to a close larger numbers of rhizoids are present on freshly collected material. While rhizoid formation is normally a feature of the basal ends of the basal cells, this secondary proliferation is often from the apical poles of medial and near basal cells and occasionally even well up in the apical region. The displacement of rhizoids from their normal position is regarded by Child (1917) as an indication of the reversal of physiological polarity and may be experimentally produced, as he showed.

Under laboratory conditions dissociation of the plant into single cells or chains of cells occurs commonly within a few days. The plant may then form new rhizoids and shoots. It is interesting that these rhizoids are often in an apical position upon the cells. While continued growth is very limited in the laboratory dissociation is regarded by Tobler (1906) as a normal means of propagation in nature.

For the purpose of the experiment a single plant was cut into small fragments which were exposed to direct galvanic current. The central part of the apparatus was a glass dish with sloping bottom to yield currents of varying intensity. The plant fragments were tied with strips of cellophane to glass cover slips and these were rested below the surface of the water upon horizontal glass rods. Sea water dripped continuously into the dish at one end and was automatically siphoned off

at the same rate at the other end   A complete change of water took place in this
way about once every hour   Lighting was obtained from a 200 watt bulb sus-
pended about a foot and a half above the plants   By means of a dish of water
and a ground glass plate, overheating was prevented and the light diffused   The
temperature was usually maintained at 22–23°C but rose occasionally as high as
26–27°C because of difficulties with water circulation   The electric current
was led into and out of the experimental dish through 2 per cent agar bridges
made up in sea water   These connected with Zn-ZnSO$_4$ electrodes   The bridges
were changed twice daily   It was found by tests with phenol red that this was
ample to prevent contamination of the central dish with acid and basic products
of electrolysis

The typical procedure is illustrated in the following experiment   The material
was collected on the morning of August 26, 1932   A medium sized sporic form
in good condition and bearing no rhizoids except upon the basal cells was cut into
eighteen fragments of 100 to 200 cells each   These were arranged in three rows
of four fragments   The depths of 1 5, 3 3, and 5 3 cm in the experimental dish
at these positions yielded current densities of 38 5, 17 5, and 10 9δ with a total
current flowing of 100 milliamperes   Six fragments were retained as controls
under identical conditions of lighting, temperature, and water   The electric
current was started at 10 30 a m   and continued without interruption, except
when the agar bridges were changed, until August 29 at 4 p m   Results were
recorded immediately after the experiment

<center>III</center>

<center>EXPERIMENTAL</center>

The following observations will serve to indicate the general nature of the results   With 38 5δ plants with apexes toward the anode showed, in the larger cells, an accumulation of chromatophores in the apex of each cell (Fig 4a)   In cells which lay across the current the accumulation was at that side of the cell toward the positive pole, and in those with bases toward the anode chromatophores had accumulated basally (Fig 4b)   A color effect, distinct from that caused by the shift in chromatophores, was also observed   In the filaments as a whole the cells toward the cathode were much more pink than those toward the anode which were paler and often of a tan shade

The most striking result of electrical treatment was the effect upon rhizoid origin   All rhizoids arose toward the anode regardless of the polarity of the cell   Cells with base toward the anode presented, therefore, a fairly normal appearance   In those oriented in the oppo-site direction rhizoids arose from the cell apexes (Fig 5)   The camera

lucida sketches (Text-fig 1) are of cells lying in various positions in the field

At the current intensity of 17 5δ results were similar in every way to those above but less marked. In those plants with apexes toward the anode the rhizoids were practically all apical in the apical region,

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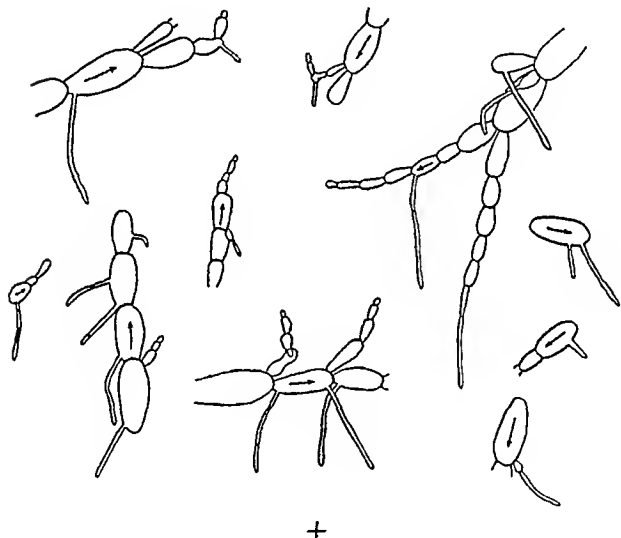
TEXT FIG 1 Camera lucida sketches of cells lying in various orientations in the electrical field to show effect upon rhizoid origin

variable in origin in the near basal cells, and in the normal basal position upon the basal cells. With 10 9δ the chromatophore shift was absent, the color change slight, and the rhizoids, as with 17 5δ, were not all electrically determined. It was interesting to note also that rhizoids were successively fewer in the 38 5, 17 5, and 10 9δ current zones

A Effect of the Electric Current upon Rhizoid Origin

In 1932 thirteen experiments of the kind described above were performed and it became evident that with proper current intensity the original polarity of the plant with regard to rhizoid formation was completely obliterated It was found that the current first became effective in this way at about 10δ and that rhizoid determination was complete between 19 and 40δ Usually in any one experiment quantitative determination proved unnecessary, it being evident by inspection that rhizoids were completely, or with only few exceptions, oriented toward the anode When the experiments are considered together it may be seen that a similar effect may be produced within a wide range of current intensity, apparently due to variability in the condition of the material In six experiments, for example, observations were made between 30 and 40δ and it was found that complete rhizoid determination occurred in four cases, a few exceptions were observed in one case, and there was no effect upon rhizoid origin in one Two experiments both showed complete rhizoid determination between 20 and 30δ Between 10 and 20δ complete determination resulted in two cases, determination with occasional exceptions in ten, and there was no effect in three Below 10δ there was no effect in any out of three cases

The influence of the electric current upon rhizoid origin was found to be so definite that few counts of rhizoids were made in 1932 In material collected late in the season there is, however, a natural tendency to produce rhizoids in an apical position upon the cells and this may confuse the results with electrical treatment As has already been pointed out, Child (1917) believed reversal of rhizoid position to be an indication of physiological reversal of polarity, and found that "inhibiting agents" were able to bring this about It has also been noted that laboratory conditions are harmful to *Griffithsia* and would be expected, therefore, especially when the material is in poor condition, to produce the same effect as specific inhibiting agents In Table I, where there is a decided tendency in the controls toward apical rhizoid production, there appears to be little determinative effect of the current except at 39δ In Table II the results of two experiments performed early in 1933 are presented The rhizoids produced by the controls were basal, with few exceptions

B Frequency of Rhizoid Production in Relation to Current Intensity

It is of obvious importance to find out whether the determinative effect of the electrical current is due simply to an inhibition of rhizoid production toward the cathode If this were the case we should expect

TABLE I

Effect of the Electric Current on Rhizoid Origin in Griffithsia
Experiment N, 1932

Apex toward +			Apex toward −	
Current density	Apical rhizoids (toward +)	Basal rhizoids (toward −)	Basal rhizoids (toward +)	Apical rhizoids (toward −)
δ				
39	All (100 per cent)	0	27	10 (27 per cent)
12	25 (62 per cent)	15	19	6 (24 per cent)
10	20 (59 per cent)	14	11	7 (37 per cent)

Controls 55 apical rhizoids (60 per cent) and 37 basal rhizoids (40 per cent)

TABLE II

Effect of the Electric Current on Rhizoid Origin in Griffithsia
Experiments 1 and 2 1933

Experiment No	Current density	No of cells	No of rhizoids			
			Toward −	Toward +	Total	Rhizoids toward +
	δ					*per cent*
1	13 0	514	6	91	97	94 0
	20 0	316	3	117	120	97 5
	30 0	277	0	180	180	100 0
	0 0 (controls)	661			134	
2	20 8	99	3	54	57	95 0
	31 2	86	1	70	71	99 0
	0 0 (controls)	175			53	

cells oriented with base toward the cathode to show no rhizoids at all However, the data show that not only are rhizoids invariably produced at the apexes of such cells, but the number of rhizoids may increase with rise in current intensity Four experiments of 1933 are pre

sented from the latter viewpoint in Table III   It may be of signifi-
cance that in the first two, performed early in the season, the rise in
number of rhizoids is most marked and in the last two, performed
toward the end of the season with material presumably in poorer
condition, the rise is slight   In the range covered by Table III there
is practically complete determination of the place of rhizoid origin
Beginning at about 40δ or somewhat below, depending probably

TABLE III

Effect of Increase in Current Density upon the Number of Rhizoids Produced

Experiment No	Current density	No of cells	No of rhizoids	Rhizoids
	δ			*per cent*
1	0	661	134	20 3
	13	517	97	19
	20	316	120	38
	30	277	180	65
4	0	175	53	30
	20 8	99	57	57 5
	31 2	86	71	79
10	0	384	52	13 5
	15	120	23	19 2
	21 7	137	27	20
	32 5	134	21	15 7
13	0	275	59	21 5
	7 9	187	31	16 7
	13	175	41	23 5
	17 8	165	39	23 7
	33 8	237	53	22 5

upon the condition of the material, the current produces injury and
eventually death

C   Polarity in Rhizoid Stimulation

It was frequently noticed that a greater number of rhizoids was
produced by plant fragments oriented with apex toward the anode
than by those oriented the opposite way   The results of three
experiments are summarized in Table IV   It may be seen that

plants with apex toward the anode produced 29 9 per cent rhizoids $\left(\dfrac{\text{Number of rhizoids}}{\text{Number of cells}}\right)$ while those with apex toward the cathode produced 18 per cent and the controls 15 4 per cent   In order to show that when the apex is toward the anode there is actually a larger amount of rhizoid material produced and that the increased percentage is not due to the production of a large number of dwarfed rhizoids, the average measured length of the rhizoids is given in the fourth horizontal row, the total calculated rhizoid material in the fifth, and the amount of rhizoid per cell in the sixth

The significance of these results is obscured by several factors Under natural conditions *Griffithsia* cells produce rhizoids at their

TABLE IV

Polarity in Rhizoid Stimulation

Effect of Orientation of Material upon Number of Rhizoids Produced   All Rhizoids Are Formed toward the Anode

	Controls	Apex toward +	Apex toward −
No of rhizoids	220	309	175
No of cells	1,428	1,033	973
Rhizoids produced per cent	15 4	29 9	18 0
Average length of rhizoids μ	1 090	1,165	1 230
Average length × No of rhizoids	244,000	337 000	213 000
Amount of rhizoid/cell	170	326	220

basal ends   With poor material collected late in the season, or material grown under the adversities of the laboratory, or after the addition of specific inhibiting substances there is a tendency toward the upset of normal polarity and rhizoids are frequently produced in apical positions upon the cells   Is the apical stimulation of rhizoids in an electrical field an indication of adverse conditions?   This does not appear to be the case   The first action of electricity is to determine the site of rhizoid origin   In plants turned with base toward the anode all rhizoids originate, without exception, at the basal ends of the cells, i e toward the anode, while in plants lying alongside but oriented the opposite way all rhizoids are apical   The action of electricity is clearly the determining factor   Therefore, the relative

stimulation of amount of rhizoid material in plants with apexes toward the anode should be regarded as a continuation of electrical action This conclusion is supported by the fact that regardless of the natural rhizoid-producing tendency of the controls which may be basal or apical, the same polar stimulation of electricity is present

D Other Data Relating to the Action of Electricity

In some experiments there appeared to be a correlation between size and frequency of rhizoids   In Text-fig 2 such a case is presented
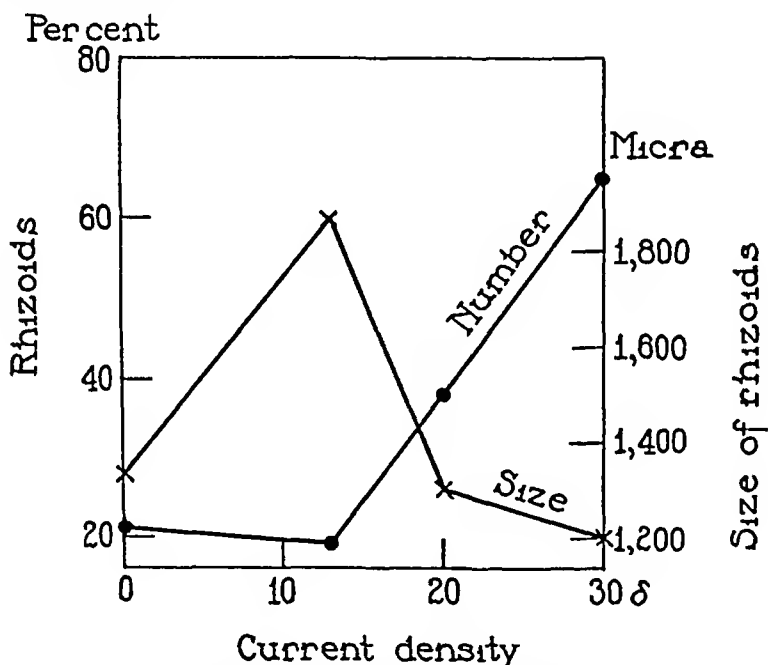
TEXT-FIG 2 Inverse relationship between rhizoid frequency and size, in the current range effective for determining rhizoid origin

Below 13δ there is neither anodal determination nor increase in number of rhizoids above the controls   This portion of the curve is therefore disregarded for the present purpose   The decrease in size which sometimes accompanies large stimulation in number with high currents suggests that a limited amount of material is available   In Text-fig 3 evidence is given that the number of rhizoids produced depends also upon the time of current action   For example, a current of 50δ acting for 24 hours had the same effect as one of 22δ acting for

72 hours These facts are in accord with the suggestion that the current acts upon some material within the cell If this is so, it should be possible by reversing the direction of the electrical flow to reverse the movement of the affected materials The following data (Experiment 18, 1933) are illustrative of attempts in this direction A current of 60δ was allowed to act for 29 hours and then reversed for 36 hours Thirty one rhizoids were produced Fourteen were
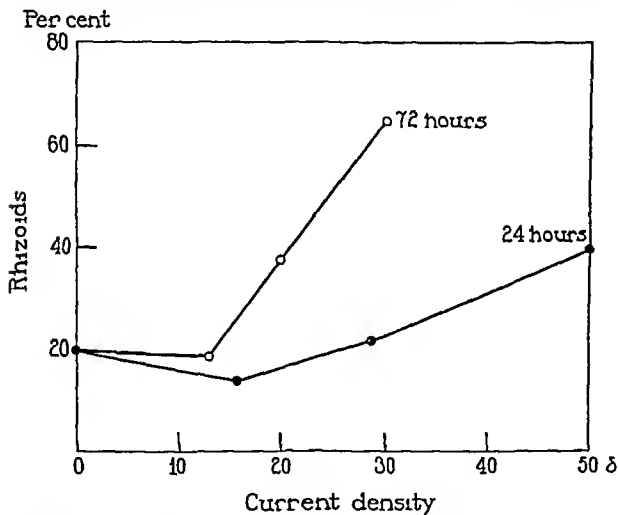
TEXT FIG 3 Increase of number of rhizoids with greater duration of electrical treatment

toward the original anode and seventeen were formed in response to the reversed current In the same experiment at 15δ, out of thirteen rhizoids only one seemed to be determined by the reversed current There were two groups of controls In one the material was treated with the current in the same direction for the duration of the experiment Thirty nine out of forty-one rhizoids were produced toward the anode In the untreated controls rhizoids were practically all

ın basal positıons upon the cells In another experıment ıt was especıally noted that after current reversal the same cells whıch have already produced rhızoıds may respond to the reversed current and wıll then show rhızoıds at both apex and base   In Table V are summarızed the results of three experiments ın whıch the current was reversed

E Effect of the Electric Current upon the Cell Contents

The coappearance of morphologıcal and electrophoretic phenomena ıs of great ınterest and possesses a certaın theoretic value ın that ıt ındıcates the actual penetratıon of the cell by sufficıent current to

TABLE V

Effect of Reversal of Current upon Rhizoid Origin

Experiment No	Current density	Time	Rhizoids toward anode	Current reversed	Rhizoids toward anode of reversed current
	δ	*hrs*		*hrs*	
18	15	29	12	36	1
	60	29	14	36	17
21	14 5	24	10	48	16
	30 6	24	6	48	43
10	24	43	49	69	5
	15	43	12	69	9
	12 5	43	22	69	10

produce dısplacement of formed bodıes   At thıs tıme, ın view of the probabılıty shown by the data that the current acts by movıng some materıal wıthın the cell, work ıs offered whıch was designed to determıne whether there ıs any causal relatıonshıp between rhızoıd determınatıon, the movement of chromatophores, and the color effect

1 Color Changes in Griffithsia Cells

Freshly collected *Grıffithsıa,* ın good condıtıon, ıs pınkısh lavender ın color   The pıgment ıs contaıned ın the chromatophores   After the plants have remaıned ın the laboratory for a few days the color fades to a pale straw, and when the cell dies ıt soon becomes entırely

green One of the criteria of good condition is color Material which is pale when collected was found to be less viable than that more brightly colored If a cell is injured by mechanical or other means a temporary flush of pink can be seen as the chromatophores release their pigment (Also described by Osterhout, 1916, 1922) On standing such cells become entirely green after a few hours, due to the escape of the red pigment to the surrounding sea water

(a) In the Electrical Field

In the electrical field, as reported above, the color of the filaments often became graded from bright reddish lavender toward the cathode to pale straw at the anode The question of course at once arose as to what this effect meant in terms of electrical action and what significance it had for rhizoid formation The work of Priestley and Swingle (1929) in which it is claimed that a causal relationship exists between a pH gradient and the differentiation of cambium into phloem and xylem and also the differentiation of shoot and root, seemed suggestive The possibility presented itself that the color gradient in *Griffithsia* represented a pH change

In view of the fact that chromatophores migrate in an electrical field it was necessary first to rule out changes in concentration of these bodies as the cause of the color effect This could easily be done The greater intensity in color appeared toward the cathode and graded off from cell to cell through the filament as a whole On the other hand, chromatophores collected toward the anode and aggregation was observed in each cell Also, with the microscope no difference in the number of chromatophores could be detected from cell to cell

(b) With Acids and Bases

In order to see whether the color changes produced electrically could be reproduced by the use of acids and bases, crude tests with high concentrations of acids (HCl, propionic, carbonic, etc) and bases (NH$_4$OH, NaOH) were made The color of the cells became altered to pink and green, respectively The following results are typical 3 to 6 drops (about $\frac{1}{4}$ cc) of N NaOH added to 10 cc of sea water containing *Griffithsia* cells resulted in a change to a bright pink color which immediately turned to green The effect was reversible

with HCl   An excess of acid left the cell bright pink   In distilled water the cells reddened distinctly within 5 minutes   1 drop of N NaOH was then sufficient to turn them green, 1 drop of HCl returned them to lavender, and 2 to 3 drops caused a pink appearance

In experiments with currents as high as 500δ in a miniature apparatus on the stage of a microscope no change in color occurred within the time of observation (? c , several hours)   Mechanical injury under these conditions resulted in the appearance after a few minutes of a brilliant pink color toward the cathode and green toward the anode The colors were like those which resulted from treatment with strong acid and base, respectively   With reversal of the current the coloration of the cell across a diameter of 0 5 mm became reversed in 2 to 3 minutes

The need for large pH changes or injury in an electrical field of relatively high intensity in order to produce color effects in the cells was puzzling in view of the changes obtained with small currents acting for several days without detectable injury   The following experiments were performed to throw light on this question

Sea water was made up with acids and bases of the kinds used above to pH 4 to 11, in steps of approximately one half of a pH unit Plant fragments were placed in these media for 3 to 4 days   In this way the exposure was comparable with that to the rhizoid-determining electric current   It was found that in the non-lethal range of 6 to 11 no color change occurred   To decide whether penetration of acid or alkali took place in the non-lethal pH range use was made of the Grubler dyes, Cresylecht violet, brilliant vital red, and brilliant cresyl violet, which were kindly given to me by Dr Josef Spek   Brilliant vital red gave a ready answer   This dye was taken up and concentrated in the cell vacuole in a short time   Stained cells placed in media of pH 6 to 11 changed color in a few minutes indicating rapid penetration   It may be suggested that, barring injury, the action of the inorganic acids and bases may be through the penetration of CO_2 which would be released by adding HCl, for example, to sea water Inorganic alkali might cause CO_2 to come out of the cell   This is an interesting point but does not alter the conclusion that the natural pigment of the cell is not responsive to these changes in pH   It is to be concluded therefore that the natural pigment of the cell is rela-

tively insensitive to changes in acidity from 6 to 11, and as the cell will not survive a more extreme range the color changes in non lethal electric currents cannot be due to changes of pH within the cell

From these experiments it seems that the following factors are responsible for the color change which appears together with rhizoid determination Chromatophores in cells toward the anode suffer a loss of pink pigment which makes these cells paler in color The pigment moves toward the cathode from cell to cell and collects in cells lying toward this pole It is suggested that the alteration in chromatophores may be of a cytolytic nature and is due perhaps to a change in salt concentration Hypotonicity by exposure to distilled water causes loss of pigment In the case of the electric current it is likely that salts are concentrated toward the anode and cytolysis may be the result of hypertonicity in this region Such change in salt concentration must come from within since the cell retains full turgidity indicating that semipermeability is unimpaired

It was shown that chromatophores of pale cells contain less pigment, by crushing under a cover slip Little pigment escaped from these while the brightly colored cells yielded a distinct flush of diffusing pigment

2 Movement of Chromatophores

With regard to the movement of chromatophores within each cell toward the anode and the relation of this to anodal production of rhizoids it was decided that chromatophore displacement by centrifugal means was the best method of attack

An electric centrifuge which carried four tubes was used The radius of rotation was 19 cm In two tubes the material was placed with the plant apexes outward, i e centrifugally, and in the alternate two in the opposite direction

In the first experiment the material was rotated at a speed of 800 to 900 revolutions per minute (approximately 150 × gravity) for 24 hours The first signs of stratification became visible after about 20 minutes After the centrifuge was stopped stratification (Fig 6) persisted for several days 6 days after centrifugation it was observed that in the material with apex centrifugally oriented many 3 celled shoots had developed Those which had been placed in the opposite

orientation in the centrifuge had few shoots and these consisted of only one or two cells   Rhizoids had developed on both sets but were larger and more numerous in those cells where the contents had been thrown basally

In another experiment these results were confirmed and the distribution was recorded (Table VI)

Where the cell bases were oriented centrifugally the average size of thirteen rhizoids was 2,250 μ, where oriented centripetally the average

TABLE VI

Effect of the Centrifuge on Size of Apical Shoots

No of cells per shoot	Material thrown into apex			Material into base		
	No of shoots	Total No of cells in shoots	Average length of shoots	No of shoots	No of cells in shoots	Length
			micra			*micra*
1	2	2	110	7	7	110
2	11	22	352	10	20	264
3	9	27	526	3	9	330
4	2	8	1,073	0	—	—
Total	24	59		20	36	

TABLE VII

Effect of the Centrifuge upon Place of Origin of Shoots

	No of plant fragments	Total No of cells	No of apical shoots	Basal shoots	Apical rhizoids	Basal rhizoids
Cell contents thrown apically	20	559	176	7	26	91
Cell contents thrown basally	18	326	64	76	27	35

size of ten was $1,440\mu$ In the controls the average length of five rhizoids was $2,250\mu$ and of ten 3-celled shoots was 930μ

These results show that with centrifugation relative inhibition or stimulation of shoots and rhizoids at the respective poles of the cells may occur   In the next experiment centrifugation was for 22 hours at an average speed of 850 R P M   Results were recorded after $7\frac{1}{2}$ days and are presented in Table VII   Fig 8 shows some of the shoots

which were displaced to the basal position and Fig 7 is of material which had been centrifuged apically

Thus both the galvanic current and the centrifuge are capable of moving chromatophores   In an electric field they collect toward the anode   Under the influence of the centrifuge they sink to the centrifugal pole   On the one hand the region of their accumulation is associated with rhizoid formation and on the other with shoot production

IV

CONCLUSION

The centrifuge and the electric current both appear to produce their morphogenetic effects by moving some material component of the cell   Doubtless, the fractionating power of these two forces is different, both in kind and in degree   In the case of the centrifuge lighter and denser materials are separated from water and substances in solution   The formation of new shoots seems associated with the region where heavier bodies are concentrated   It may be of some significance in this relation that differentiation of menstematic cells and the accompanying loss of formative power normally appear to be associated with dilution of the protoplasm and the formation of large water vacuoles   Optically at least, the centrifuge reverses this process and it may act as a mechanical means of moving water away from the centrifugal, shoot producing pole   The possibility is therefore suggested that water removal and protoplasmic concentration may be the mechanism responsible for the results noted in the centrifuge experiments   The activation of cells by plasmolytic removal of water seems to be a comparable case, and this has been demonstrated by Kreh (1909) with liverwort thalli, Miehe (1905) with *Cladophora*, Borger (1926) with mosses and ferns, and Prat (1932) with some marine algae

With regard to the movement of chromatophores in an electrical field the response may be similar to the movement of plastids under the influence of light and not due to electrical motive power at all   The evidence seems to point to an actual electrophoresis, however, in that the movement of chromatophores is more marked with increase in current intensity up to and beyond the lethal point   In tactic

phenomena such as response to light there is always, so far as I am aware, a point of maximal response sometimes followed by reversal with increased stimulation   The conclusion that the chromatophores are moved by electrical forces is important in that it indicates the penetration of the cell by sufficient current to produce electrophoretic phenomena

Any alteration in acidity in the cell would be of great significance because of the effect upon the viscosity and activity of the protoplasm Kuhne's (1864) interpretation of electrically produced color changes in *Tradescantia* cells as due to pH has been widely accepted, but the recent work of Blinks (1932) indicates that a migration of the normal pigment is responsible for the observations   Similarly, it has been shown in this paper that alteration of pH, although comparatively small changes have not been ruled out, cannot account for the color changes observed in *Griffithsia*, as only by the addition of lethal quantities of acid or alkali could the color effects be duplicated   It has been shown, moreover, that the red pigment of the chromatophores can be released in large quantity by mechanical injury and that the released pigment migrates toward the cathode   This is sufficient to account for the color changes, and has other significant aspects, as follows

The observed migration of small amounts of pigment in living cells corroborates the conclusion that sufficient current traverses the cell to produce appreciable electrophoresis   The loss of pigment by chromatophores toward the anode seems explicable on the ground of changes in concentration of salts   Salt migrations under electrical influence are usual in inanimate systems and have also been postulated in living cells   Scheminzki (1924), for example, accounts for anodal precipitation of proteins in trout eggs on the basis of loss of cations and the consequent effect upon the stability of the protein solution   It is suggested that some such phenomenon leading to impairment of the surface film of the chromatophores is responsible for loss of pigment

The movement of chromatophores toward the anode, the movement of released pigment toward the cathode, the release of pigment by chromatophores at the anodal ends of the filaments together with the direct relationship of current intensity to the number of rhizoids

produced, and the reversibility of the effects of the current here reported, all point to a causal relationship of rhizoid production in the electrical field to the movement of some material   The results with the centrifuge make it quite unlikely that the chromatophores themselves are responsible   It seems certain that less obvious migrations are involved, and Hardy (1913) has shown that electrical displacement of granules may take place in living material   Some very suggestive work has been done by Spek (1933) on various invertebrate and vertebrate eggs   Dr Spek believes that granules of various kinds and the medium are capable of maintaining individual pH   Such organization of the protoplasm, he says, introduces wide possibilities for cataphoresis when we reflect that this system of differently charged particles is bounded by membranes in which definite potentials have been abundantly demonstrated   Cortical differentiation can be explained at once in the sense that some particles will be attracted to and others repelled by the membrane, and with a heterogeneous membrane bipolar differentiation can also be accounted for

If the differentiation of egg cells is an electrophoretic phenomenon due to the migration of charged particles under the influence of membrane potentials, may we not, in view of the evidence contained in this paper assume a similar mechanism for the differentiation of rhizoids under the influence of an externally applied current?

v

SUMMARY

1 Direct galvanic current of 10 to 40 microamperes per square millimeter of cross section of medium results in anodal determination of rhizoid origin in the differentiated cells of the red alga *Griffithsia borneliana*   The current is most effective near the upper end of the range

2 Within the range used there is an increase in the number of rhizoids produced with increase in current intensity and a decrease in size of rhizoids

3 Currents of lower intensity require a longer time to produce these effects than comparatively high currents

4 The orientation of the plants in the electrical field seems to affect

the number of rhizoids produced, in that plants with apexes toward the anode produce more rhizoids

5 Together with anodal rhizoid determination there is migration of chromatophores toward the anodal side of each cell

6 Displacement of chromatophores (and other cytoplasmic bodies) by the centrifuge does not affect the point of rhizoid origin, but does affect the shoots

7 Together with anodal determination of rhizoids the algal filaments become graded in color, from bright pink toward the cathode to pale tan toward the anode

8 Evidence is presented to show that this is not due to a pH change, but to a loss of pigment by chromatophores toward the anode and electrophoresis of the pigment toward the cathode

9 In conclusion the probability is pointed out that the current acts in morphogenesis by moving particles of different charge

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EXPLANATION OF PLATES

PLATE 1

FIG 1 Early cleavage of *Griffithsio* spores *a*, beginning to elongate before first cleavage *b*, rhizoid formed *c*, polarity completely established *s*, shoot, *r*, rhizoid

FIG 2 Apex of mature plant

FIG 3 Base of plant with rhizoids in normal position on cells

FIG 4 Anodal accumulation of chromatophores *a*, apex toward anode *b*, base toward anode

PLATE 2

FIG 5 Rhizoids in apical position toward anode *a* apex of cell *b* base

FIG 6 Effect of centrifugation on cell contents *a*, apex centrifugally oriented. *b*, base centrifugally oriented

FIG 7 After centrifugation Apex centrifugally oriented Shoots in normal position

FIG 8 After centrifugation Base centrifugally oriented Shoots from cell bases *a*, apex of cell, *b*, base
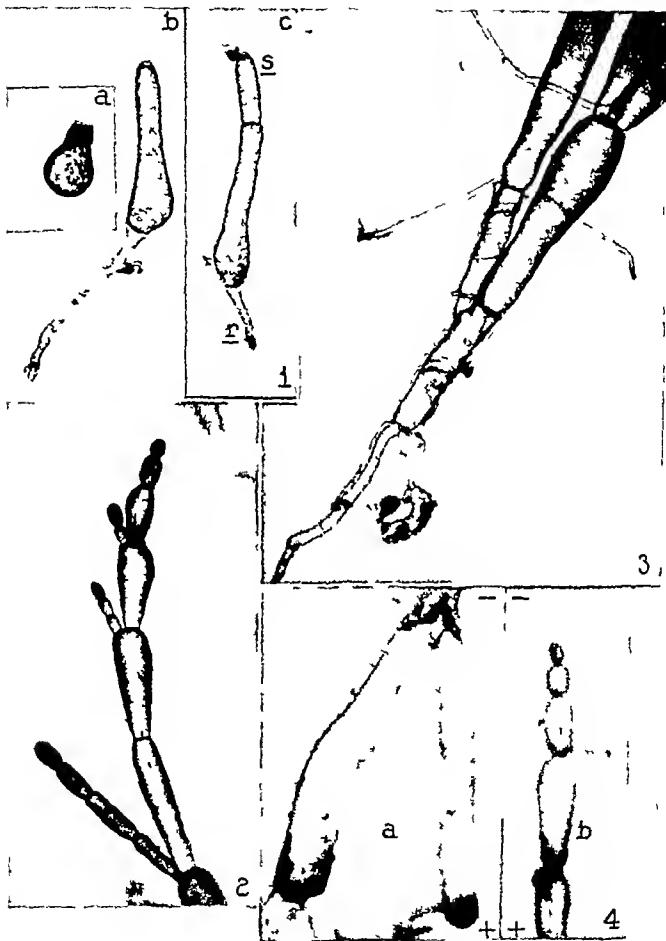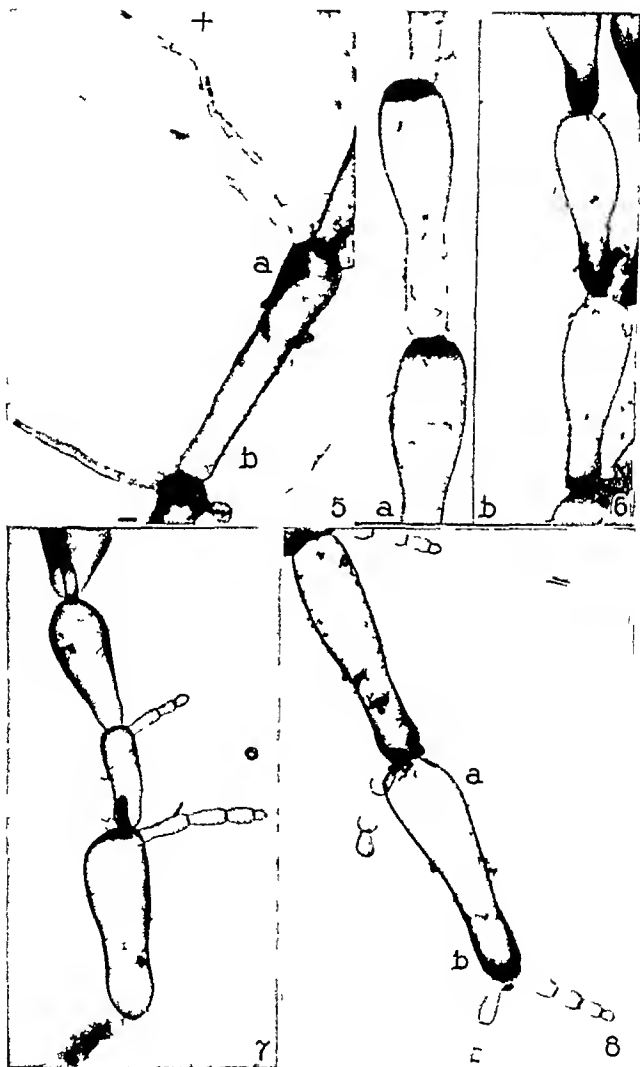
PLATE 2

(Schecht r Electrical cont ol of rh d f rmati n)

STUDIES ON THE GROWTH HORMONE OF PLANTS

VI The Distribution of the Growth Substance in Plant Tissues

By KENNETH V THIMANN

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Recent studies on the growth hormone of plants have indicated its very wide distribution in both the plant and animal worlds   Its function in promoting growth by cell elongation, while so far as we know confined to plant tissues, is, nevertheless, of a completely non-specific nature   Coleoptiles of Gramineae, stems of Commelinaceae, flower stalks of Compositae, stems of Leguminosae and the gynostemia of some orchids are all subject to the influence of the hormone, and, so far as cell elongation is concerned, are probably completely dependent upon it   Nevertheless, the presence and quantity of the growth hormone in many plant tissues is difficult to establish, largely because, although soluble in water, the substance appears not to be extracted on grinding the tissues with water   Thus, Went (1928) was able to extract growth substance from coleoptiles of *Avena* by placing them on agar but not by grinding with water   In the course of various attempts to determine the growth substance in plant tissues, it was found that in general only small amounts could be extracted on grinding with water, while extraction with organic solvents often yielded considerable amounts of the hormone

Furthermore, it was shown in Part I of this series (Dolk and Thimann, 1932) that the growth hormone is a relatively strong acid, of pK = 4 75, and hence it follows that in order to extract it in organic solvents the aqueous tissue must be brought to a hydrogen ion concentration of at least 10^{-2} It must also be remembered that the amount of growth hormone which will produce a curvature of 1° in a standard *Avena* coleoptile when present in an agar block of volume 10 7 mm^3 (= one plant unit) is only 1/200 of the amount which must be present in 1 cc of liquid, so that in order to detect the minute amounts of growth

substance present in coleoptiles and similar tissues, the extracts must be made up to as small a final volume as possible

These considerations led to the following technique for extraction The fresh material is killed by immersing in chloroform, about one-fifth of its volume of 1 N HCl is added, and the mixture thoroughly ground   The chloroform layer is separated off, and the acidified tissue ground twice more with chloroform   Ether is not used on account of the destructive effect on growth substance of the traces of peroxide always present in ether (cf Dolk and Thimann, 1932)   Smaller amounts of peroxide may also be present in chloroform particularly after exposure to light, but these amounts are usually too small to produce any appreciable inactivation   Finally, the chloroform is evaporated off and the lipoidal material taken up in a very small volume of water, usually 0 3 cc , and tested by adding an equal volume of 3 per cent agar, pipetting 0 5 cc of the mixture into

TABLE I

Comparison of Water and Chloroform Extractions

Material	Growth substance in plant units per gm or piece		Ratio Growth substance in CHCl₃
	CHCl₃ extraction	H-O extraction	Growth substance in H₂O
Wheat germ	320,000 per gm	31,000 per gm	10
Pollen of *Sequoia*	4,000,000 per gm	160,000 per gm	25
Terminal buds of *Vicia faba*	46 1 per bud	9 6 per bud	5
Coleoptile tips of *Avena*	5 8 per tip	4 7 per tip	1 2
Coleoptile bases of *Avena*	6 9 per base	1 3 per base	5

the standard brass ring, and cutting into blocks of volume 10 7 mm ^{3} The amount of growth substance which must be in such a block to give a curvature of 1° is one plant unit, which is equal to 0 4 × the *Avena* unit of Kogl, Haagen-Smit, and Erxleben (1933)

Table I compares the amount of growth substance obtainable in this way from a few representative plant tissues with the amount set free by simple extraction with water   The water extractions were made by adding acid as before and grinding as thoroughly as possible three or four times with small volumes of water   It may be noted that by this method the pollen of *Sequoia* is seen to be an even better source than the pollen of orchids used by Laibach (1933)   The growth substance obtainable from buds of *Vicia* is approximately

equal to the amount which would diffuse out into agar in 1 hour, as determined in a previous communication (Thimann and Skoog, 1933) This indicates that the growth substance would occupy 1 hour in travelling from the extreme tip of the bud to the cut surface Since this distance is about 1 cm , the velocity of movement of the growth substance is 1 cm per hour, a figure which agrees with the determinations of Van der Wey (11 mm per hour in Experiment 80 and 10 mm per hour in Experiment 79, 1932)   The ratio in the last column

TABLE II

Inactivation of Growth Substance by Leaf Extract
Time of contact between growth substance and leaf extract about an hour

Concentration of growth substance solution used	Activity in units per cc.		Ratio $\frac{A_{H_2O}}{A_{LE}}$
	In water	In leaf extract	
(a) *Vicia faba* Catechol oxidase present			
a	40 2 *	8 8	4 5) 4 4
a/2	20 1 *	4 7	4 3)
(b) *Helianthus annuus* Catechol oxidase present			
b	15 6	4 7	3 3)
b	15 6	4 4	3 5) 2 8
b	15 6	6 5	2 4)
b/2	7 8	3 7	2 1)
(c) *Malva parviflora* Catechol oxidase absent			
a/2	20 1 *	13 5	1 5) 1 3
a/4	10 0	8 8	1 1)

* Assay at higher dilution

shows that, with the exception of coleoptile tips, the chloroform extraction yields at least five times as much growth substance as water extraction

In order to prove that the low yields obtained on extraction with water are due to inactivation of the growth substance by plant enzymes the following experiment was carried out   A leaf extract was made by crushing 5 gm of *Vicia faba* leaves in a little water and making up to 4 cc   A concentrated growth substance solution was then diluted

with this extract and with water in parallel experiments and the two solutions tested   The results, summarized in Table II (*a*), show that the leaf extract causes a great inactivation of the growth substance and that the ratio of activity in leaf extract to activity in water (last column of the table) is of the same order as that between chloroform and water extracts, as given in Table I   A leaf extract made from *Helianthus annuus* gave similar results (Table II (*b*))   It has also been reported, but without details, by Kisser *et al* (1931) that the addition of crushed leaves to a growth substance solution resulted in inactivation

Since it was shown in Part I of this series (Dolk and Thimann, 1932) that the activity of the growth substance was destroyed readily on oxidation, it seemed probable that the inactivation by leaf extract is due to oxidation by the peroxidase-catechol-oxidase system, which is widely distributed among plants   If this is so, then a leaf extract made from a plant which does not possess this system should not cause inactivation [1]   This was proved by an experiment, carried out exactly as above, but using an extract from the leaves of *Malva parviflora*   It was shown by Onslow (1921) that the Malvaceae are lacking in this enzyme system   The results, in Table II (*c*), show almost no inactivation by this extract   Hence, the inactivation is probably an oxidation, and since the majority of plants contain this or a related oxidase system, it follows that as a general procedure the grinding of plant tissues with water is undesirable, as it will lead to the oxidation of the growth substance contained in them   This fact provides an explanation for the results of Cholodny (1931), who showed that after wounding, the sensitivity of the wounded side of *Lupinus* hypocotyls to light and gravitation was greatly reduced   This phenomenon is thus to be ascribed to enzymic inactivation of growth substance and not necessarily to a special wound hormone

The Distribution of Growth Substance in Avena Coleoptiles

Since the placing of coleoptiles on agar after they have been decapitated yields no growth substance, it has generally been assumed that

[1] The dihydroxyphenylalanine oxidase system of *Vicia faba* is to be regarded as a special form of the catechol oxidase

the substance is used up in the growth reaction and disappears from the coleoptile   For studies on the mechanism of the action of the hormone, it is desirable to know whether it really disappears or not from the tissues in which it has reacted   The above simple method of extraction, while it cannot determine whether or not the substance is used up in the growth reaction itself, can at least determine whether growth substance is present in the lower parts of the coleoptiles and if so to what extent

It has already been shown (Table I) that the substance is, in fact, present in detectable amounts in the bases of the coleoptiles   Table

TABLE III

Growth Substance Extractions from Avena Coleoptiles

	No. taken	Length	Volume of final solution	Curvature	No. of test plants	Growth substance per piece in plant units	Growth substance per unit length in plant units per mm	Average plant units per mm
		mm	cc					
Tips	90	5	0 5	4 6	11	5 2	1 04	
	128	5	0 4	9 3	19	5 8	1 16	
	99	5	0 5	3 5	17	3 5	0 70	
	166	5	0 6	19 6 *	35	7 2	1 45	
	89	5	0 6	4 6		3 1	0 61	0 99
Bases	128	15 av	0 4	11 1 *	21	6 9	0 46	
	166	22 av	0 9	35 2 *	52	12 7	0 58	
	89	22 av	0 6	7 3°	26	9 9	0 44	0 49

* The angles in these instances were determined by diluting the agar blocks according to the method of Went (1928)

III summarizes a number of determinations made on tips and bases   In order to clarify the details of the method, all the measurements are given in full   The last column shows that the number of plant units per millimeter of coleoptile length is fairly constant, and the amount in the base is unexpectedly large, being about half that in the tip

A closer analysis of the distribution was made by dissecting a large number of coleoptiles of the same age, the primary leaf being removed, the sections extracted were as follows   (1) the topmost 2 mm , (2) the

next 3 mm , (3) the next 3 mm , (4) the next 5 mm , and (5) the re-
mainder   The total length of these remainders was measured and
the average obtained   The results of four such experiments are given
in Table IV   While there is marked variation in the absolute amounts
which is no doubt due to the extremely small quantities dealt with,
the type of polar distribution is the same throughout   In Fig 1 the
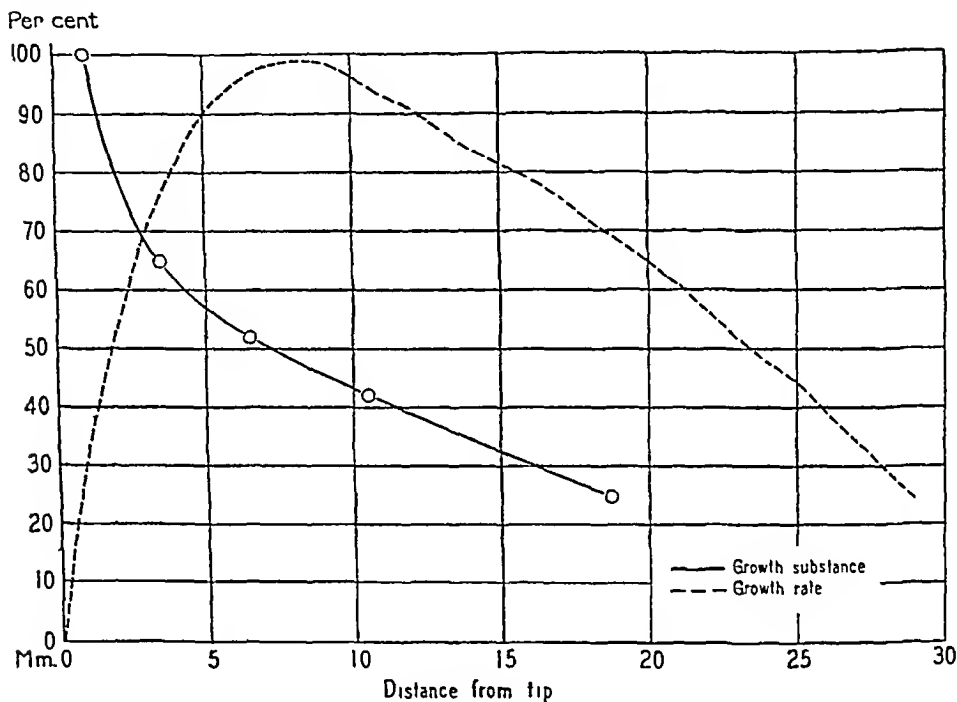
FIG 1 Distribution of growth substance and growth rate in the *Avena* coleop-
tile   Solid curve, growth substance as per cent of the amount in the 2 mm tip,
mean of four experiments in Table IV   Broken curve, growth rate as per cent
of the maximum rate, mean of four experiments by F W Went (three unpub-
lished)   Average length of coleoptiles extracted, 26 mm

mean distribution, in per cent of the amount in the tip, is contrasted
with the distribution of growth rates as given by Went   While the
amount of growth substance per millimeter falls off steadily with dis-
tance from the tip, the rate of growth, as is well known, passes through
a well defined maximum   There is no accumulation of growth sub-
stance in the middle of the coleoptile, and the view of Went that an-
other factor limits growth in the apical portion is thus borne out

The amount of growth substance diffusing out from tips when placed on agar was determined for comparison   It proved to be about five plant units per tip per hour, and to be maintained for at least 6 hours There is therefore no doubt that the tip continues to produce growth substance when cut off, since by extraction only about 1 hour's supply can be obtained   From these experiments it follows that the growth substance does not immediately disappear from the tissues   On the other band, since cut off sections of coleoptile continue to grow for some time, it cannot be argued that the growth substance which is extracted by chloroform bas already taken part in the growth reaction It was shown by Bonner (1934) that decapitated coleoptiles still con-

TABLE IV

Distribution of Growth Substance in the Coleoptile

Section No	Length	Experiment 1		Experiment 2		Experiment 3		Experiment 4		Average as per cent of plant units per mm. in tip
		Plant units per section	Plant units per mm	Plant units per section	Plant unit per mm.	Plant unit per section	Plant unit per mm.	Plant units per section	Plant unit per mm	
	mm									
I	2	4 91	2 46	1 66	0 83	1 12	0 56	1 33	0 67	100
II	3	—	—	1 40	0 47	1 35	0 45	1 33	0 44	65
III	3	5 20	1 73	1 04	0 35	1 22	0 41	0 92	0 37	52
IV	5	2 82	0 56	1 20	0 24	1 50	0 30	1 66	0 33	42
V	Base	4 70	0 34	1 34	0 11	2 87	0 37	1 13	0 09	25
Average length of coleoptile		27 0 mm		25 6 mm.		22 2 mm.		25 8 mm		

tain enough growth substance to give a curvature when acid is admitted to the tissues   It seems therefore that the growth substance is present in two different forms, one, as in tips of coleoptiles, in which it diffuses out into agar, and one in which it does not, although it is still extractable from the tissue   Tentatively it may be suggested that the first, or free, form is the one which is redistributed in the coleoptile under the influence of light or gravity and is thus responsible for tropisms   The growth substance which is set free from its salts by the action of acid, as in the experiments of Bonner, is principally in the second or bound form, since it does not diffuse out of the coleoptile, but is obtained by extraction

Distribution of Growth Substance in Roots

Some conflict of opinion has arisen in regard to the presence of growth substance in roots   Thus Gorter (1932) was unable to confirm the work of Cholodny (1928) and others that decapitated roots will show a geotropic response if root or coleoptile tips be applied under the right conditions   While the conflict is largely resolved by the recent paper of Cholodny (1933), no conclusive evidence has been brought forward to show whether growth substance is produced in the root tip or not However, Boysen-Jensen (1933), by using dextrose agar, has been

TABLE V

Distribution of Growth Substance in Avena Roots

Section	Total length of combined sections	Angle	No of plants	Total No of plant units	Plant units per mm	Per cent
	mm					
(1) 1st 10 mm	1500	5 1°	19	306	0 204	100
(2) Base	5250	12 7°	23	762	0 145	73
(1) 1st 10 mm	1410	8 5°	18	510	0 362	100
(2) 2nd 10 mm	1410	7 2°	17	432	0 306	84 5
(3) Base	3713	$\begin{cases} 13\ 5° \\ 7\ 5 + 8\ 2° \end{cases}$	$\begin{cases} 11 \\ 10 \end{cases}$	876	0 235	65
(1) 1st 10 mm	1240	$\begin{cases} 14\ 7° \\ 2 \times 8\ 6° \end{cases}$	$\begin{cases} 11 \\ 12 \end{cases}$	960	0 774	100
(2) 2nd 10 mm	1250	13 1°	18	786	0 629	81
(3) Base	3550	$\begin{cases} 2 \times 11\ 5° \\ 4 \times\ 5\ 9° \end{cases}$	$\begin{cases} 11 \\ 11 \end{cases}$	1400	0 394	51

able to cause growth substance to diffuse out from roots of *Zea*, and has shown that the amount so obtained decreases steadily with increasing distance from the tip   However, his experiments, while very valuable, still do not show whether it is the actual concentration of growth substance present, or only the ease with which it diffuses out, which decreases with distance from the tip   If it could be shown by direct extraction that the concentration really increases towards the tip this would go far towards settling the question   Such experiments were therefore undertaken

The roots of *Avena*, grown in water in the usual way, were rubbed in running water to free them from adhering bacteria, etc , and dissected into three portions, the tip 10 mm , the second 10 mm , and the basal part, which varied in length from 10 to 40 mm A large number of these were obtained at one time and extracted The results, Table V, show not only that growth substance is present in roots, but also that its distribution is polar, *i e* , that its concentration is greatest at the tip When it is further considered that the apical end of the root tapers somewhat, the amount of growth substance per unit of weight would show an even more marked polar distribution The concentration of

TABLE VI

Growth Substance Diffusing out of 10 Mm Root Tips into Dextrose Agar

No of roots	Time on agar blocks	No of blocks	Angle	Plant units per root	Control
	hrs				
40	1 5	12	2 7	0 8	+1 0
20	3	6	4 0	1 0	
20	12	6	2 3	0 7	
24	18	12	5 1	2 6	
20	23	12	5 3	3 2	0 0
36	24	6	16 5	3 5	
21	46	12	6 4	3 7	
36	48	12	11 1	3 7	
30	72	12	6 5	2 6	+1 1°
20	72	12	4 7	2 8	−2 0
By extraction, mean of three experiments in Table V				4 35	

growth substance per unit length is somewhat less in the root tip than in the coleoptile tip, but in the root base it is about the same as in the coleoptile base The extent of the decrease with distance from the tip is comparable to that in the coleoptile, and would seem to indicate that growth substance is produced in the tip

On the other hand, if growth substance is produced in the root tip then it should be possible by diffusion to obtain it in larger amounts than can be found on direct extraction Thus it was shown above that the amount of growth substance extractable from coleoptile tips, 3–5 plant units, is produced hourly for at least 6 hours when the tips

are placed on agar   Since Boysen-Jensen has shown that growth
substance diffuses out of root tips in a similar way if agar containing
10 per cent dextrose is used, experiments were carried out in which
Avena root tips were placed on this agar   The roots were first well
washed as before, and a number were lightly clamped together between
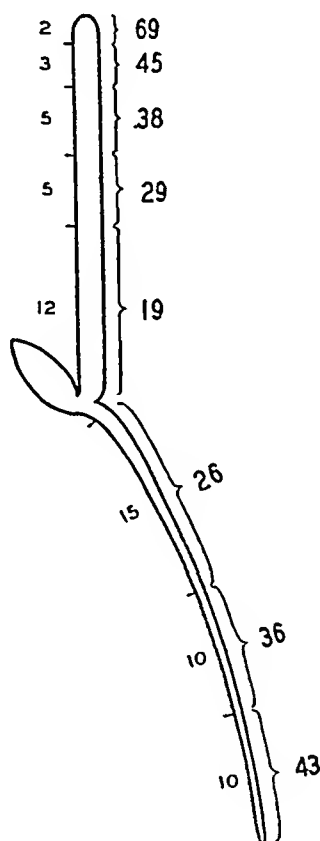
FIG 2 Distribution of absolute growth substance concentrations in plant units
per millimeter in the *Avena* coleoptile and root   The small figures indicate the
lengths of sections used in millimeters

pieces of cork, cut off at 10 mm from the tip, and pressed gently upon
the dextrose agar, contact being insured by means of a thin film of
water   Diffusion took place in a moist chamber in the dark at 25°,
and at the end of the experiment the root tips showed no drying out
and appeared in good condition

Confirming Boysen-Jensen's results, growth substance was readily

obtained in this way, the amount increasing with time of contact as shown in Table VI However, in no case did the amount of growth substance exceed that obtainable by direct extraction, but on the con trary it appeared to approach that amount asymptotically   After 48 hours' diffusion a maximum was reached, after 72 hours the amount was somewhat less (Column 5, Table VI), possibly due to decomposi tion by microorganisms   Control agar blocks left in the same chamber for the same period did not develop any growth substance   Thus root tips behave in the opposite way from coleoptile tips and do not con tinue to produce growth substance when cut off

This being the case, the only fair conclusion seems to be that growth substance is not produced in the root tip but merely accumulates there, being brought there by the polarity of its transport   This may ex plain why the presence of dextrose in the agar is necessary to draw it out, an osmotic gradient being thus set up which causes the growth substance to diffuse backwards   While in conflict with certain experi ments in the literature, this view, based on direct growth substance determinations, seems to the author unavoidable   An alternative is that production of growth substance in the root tip, being dependent upon the supply of a precursor from the seed or plumule, ceases when this supply is cut off

The results of the distribution experiments are summarized by Fig 2, which expresses the growth substance in plant units per mm in coleoptile and root   That the amounts in coleoptile base and root base are similar makes the above view reasonable

SUMMARY

1 It is shown that when plant tissues are ground with water the growth substance contained therein is inactivated by the oxidizing enzymes

2 A simple method of extraction is described which enables the quantitative determination of growth substance in such tissues

3 The amount and distribution of growth substance in the *Avena* coleoptile is determined by this method, and it is shown that while the substance does not diffuse out from the lower parts of the coleop tile, it is nevertheless present in considerable amounts, the concentra tion decreasing steadily with the distance from the tip

4 Growth substance is also present in considerable amounts in *Avena* roots, and here also its concentration decreases steadily with distance from the tip

5 The amount of growth substance diffusing out of root tips into dextrose agar, even during long periods of time, is not greater than the amount obtainable by direct extraction   Actual production in the root tip therefore either does not take place at all, or else takes place under quite different conditions from the production in the tip of the coleoptile

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CRYSTALLINE ACETYL DERIVATIVES OF PEPSIN

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The isolation and identification of several individual enzymes has made it possible to attack the problem of their chemical structure   In the last few years it has been shown that urease (1), pepsin (2), trypsin (3), chymo trypsin (4), probably pancreatic amylase (5) and rennin (24) are proteins, so that the general molecular structure of these enzymes is known   Enzymes have powerful catalytic properties, however, not possessed by ordinary proteins and therefore must possess characteristic chemical structures

Since the chemical properties of a molecule are not the sum of the properties of its component parts, the catalytic activity of an enzyme must be considered as a property of the whole molecule and cannot, strictly, be referred to one special group   It is frequently true, however, that a particular property of the molecule is dependent upon a particular group or arrangement of the atoms within the molecule, the magnitude of this property depends upon the structure of the whole molecule   Thus organic acids usually contain a carboxyl group but the strength of the group depends on the entire molecular configuration   In the case of proteins possessing peculiar properties the first question to arise is whether the molecule consists entirely of amino acids or whether it contains a prosthetic group, *i e* , a compound not composed of amino acids   The peculiar properties of hemoglobin are ascribed to the presence of the prosthetic group, heme, which can easily be separated from the protein, globin   In this case the prosthetic group may be detected by its color   The yellow respiratory ferment of Warburg (6) is similar since again the prosthetic group is colored   In both these cases the characteristic properties are lost completely when the prosthetic group is separated from the rest of the molecule   The prosthetic group alone is therefore not responsible for the activity

The products of hydrolysis of pepsin have no proteolytic activity and there is no other property known of the hypothetical group which in pepsin renders it active   Indirect information as to the nature of the active group may be obtained, however, by altering the structure of the original pepsin molecule with reagents known to react with certain chemical groups and determining the effect of this change in structure upon the activity   By analogy with other compounds, such as acids or dyes, it would be expected that any change in the molecule would affect the activity but that the extent of the effect would depend upon a number of factors such as the nature of the reagents, the reacting groups of the molecule, and their relation to the active part of the molecule   Pepsin is known to have such free groups as carboxyl (COOH), amino (NH_2), and phenolic hydroxyl (OH)   The effect on the activity resulting from the modification of these groups should furnish information as to their relation to the molecular structure responsible for the activity   If the modification of these groups in native pepsin resulted in a complete loss in activity it would be probable that the active group, or one closely associated with the active group, had been altered by the treatment   If the modification of these groups failed to affect the activity it would follow that the modified groups are not closely related to the active part of the enzyme   The present experiments were designed to determine what effect acetylation of the various groups of pepsin would have on the activity

The action of a number of biological substances is markedly altered by the introduction of one or more acetyl groups into the molecule   Hunt (7) has shown that acetyl choline has at least 40,000 times as much action on blood pressure as does choline   Warburg and Negelein (8) have prepared a diacetyl deutero hemoglobin which has only 52 per cent of the capacity to combine reversibly with oxygen as does ordinary hemoglobin   Workers in several laboratories have studied the change in properties of crystalline insulin after acetylation   Freudenberg and his coworkers (9) have reported complete inactivation of crystalline insulin by acetylation   Part of the activity could be recovered by treatment of the inactive acetylated insulin with dilute alkali   Later, using freshly precipitated amorphous insulin, they reported a product of acetylation which had 5–10 per cent of the original activity   The amino nitrogen was reduced from 1 per cent to 0 1 per cent by the acetylation   More recently Avery and Goebel (10) have reported that an acetyl group present in the Type I specific pneumococcus polysaccharide renders the material antigenic   This property disappears on removal of this one acetyl group

Hugounenq and Loiseleur (11) have reported that treatment of crude pepsin solutions with methylating and diazotizing agents failed to affect the proteolytic activity of the enzyme. Their treatment was directed toward the primary amino groups and since they noticed no change in activity of the enzyme they concluded that the primary amino group had little if anything to do with the proteolytic activity. They did not however, measure either the loss in amino groups nor increase in methyl groups, nor did they isolate any compounds

In the present experiments pepsin was acetylated by treatment with ketene and it was found that the activity decreased as the acetylation proceeded. In the study of the course of the acetylation reaction it became apparent that the time rate of change in the specific activity of the enzyme during acetylation depends upon the pH of the solution. Acetylation of pepsin at pH 5 5 causes a greater drop in specific activity per acetyl group introduced than acetylation at pH 4 5. If the pH of the pepsin solution is kept constant the specific activity of the reaction mixture when plotted against the increase in number of acetyl groups per molecule of protein drops off logarithmically as acetyl groups are introduced. There is no sharp break in the curve, the activity drops gradually and is 10 per cent or less of the original activity after the introduction of more than twenty acetyl groups per molecule of enzyme.

A preparation of acetylated pepsin has been obtained in crystalline form which has 60 per cent of the activity of the original pepsin, as measured by several methods. This material contains one or less primary amino groups per molecule of protein instead of the three or four originally present in pepsin. It also possesses from six to eleven acetyl groups per molecule, the number depending upon the conditions during the acetylation. From the acetyl determinations of several different preparations and the solubility experiments it appears that there may be several acetyl products possessing approximately 60 per cent of the original pepsin activity and which may form solid solutions with one another.

Another crystalline acetylated pepsin preparation was obtained by subjecting the above acetyl pepsin to normal sulfuric acid. The specific activity of this acid solution increased to the value of pure pepsin and the acetyl content dropped from nine to four acetyl groups per molecule. There was no measurable increase in the

primary amino nitrogen and it is probable that the remaining acetyl groups are on the primary amino groups   It was also possible to prepare this same, or a closely related material, starting with pepsin By careful acetylation an acetyl product was obtained which had lost none of its activity but which had lost all of its primary amino groups This material had three acetyl groups per molecule, the equivalent of the amino groups lost

Still another acetylated product was prepared by acetylation for 35–40 hours at pH 5 5 in a dialyzing bag   This material was only 10– 15 per cent as active as pepsin and had on the average twenty acetyl groups per molecule   On further acetylation the decrease in activity was very slow   It was much less soluble than the other preparations and crystallized with difficulty and in very small crystals

The different acetyl preparations could be separated from one another by fractional precipitation from concentrated magnesium sulfate solution   Under the microscope the crystals of all of the acetyl preparations appeared alike and indistinguishable from those of pepsin as may be seen in Fig 1

The results of these acetylation experiments show that acetylation of primary amino groups does not change the specific activity of pepsin by more than 10 per cent but that addition of acetyl groups in some other places causes a marked decrease in activity

The primary amino groups of proteins in general are those of lysine and since acetylating these groups has little or no effect on the activity it is probable that the lysine groups are not part of or are not intimately associated with the molecular structure responsible for the activity   Further acetylation, however, causes a marked decrease in activity which would indicate that certain groups of the protein more closely related to the active part of the molecule have been acetylated Ketene reacts with NH, OH, or COOH groups and it is not possible to decide at present between the various possibilities   The ease with which these groups may be removed suggests that OH, or COOH groups have been acetylated

The pH activity curves, acid and alkali inactivation, and the titration curves of the acetyl preparations examined were not greatly different from the same properties of the parent substance, pepsin On the other hand no isoelectric point could be detected for the "100
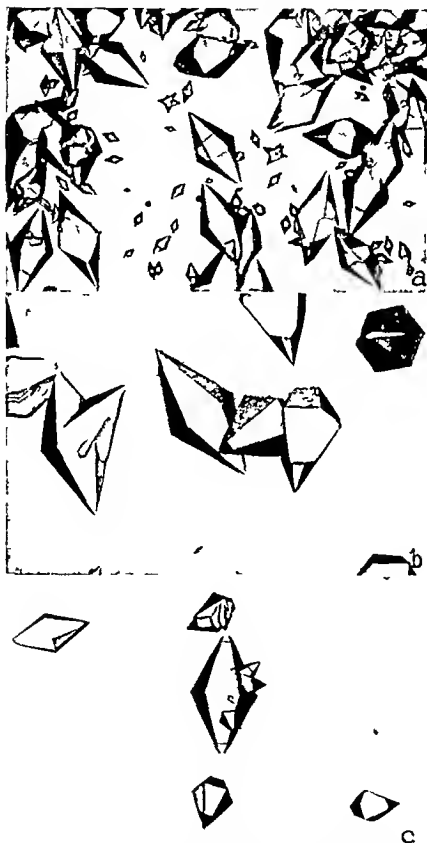
FIG 1 (a) Crystalline pepsin (b) Crystalline 100 per cent active acetyl
pepsin (c) Crystalline 60 per cent active acetyl pepsin

per cent active" and "60 per cent active" acetylated derivatives and their solubilities in acid decreased as the concentration of acid increased   These two properties are quite different from those of pepsin which has an isoelectric point at pH 2 7 and the solubility of which increases as the solution is made more acid than pH 2 7

EXPERIMENTAL RESULTS

Acetylation with Ketene

Staudinger (12) in his study of ketene ($CH_2 = C = O$) pointed out that ketene is capable of reacting in dilute acid but the reaction is more rapid in alkaline solution and that whereas it reacts with all of the following groups—primary and secondary amines, aliphatic alcohols, water and phenols—the rates of reaction are quite different for each group   Primary amines react most rapidly and the phenols least rapidly   The reactions of ketene are in general addition reactions and polymerizations, the latter being of little or no consequence in this work   The addition reactions may be illustrated as follows

$$CH_2 = \underset{\underset{O}{\|}}{C} + H - NH - R \longrightarrow CH_3 - \underset{\underset{O}{\|}}{C} - NH - R$$
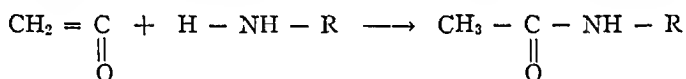
Acetylation of amino acids using ketene has been reported by Bergmann and Stern (13)   This method is more suitable for the acetylation of pepsin than the usual methods since the reaction may be carried out in slightly acid aqueous solution and without any appreciable denaturation of the protein

The ketene used in this work was generated by the thermal decomposition of acetone vapor (14)

Course of the Acetylation Reaction

Effect of pH —In the early experiments it was noticed that when ketene was passed into a 5–10 per cent pepsin solution in 3 M pH 5 5 acetate buffer, the specific activity of the enzyme dropped rapidly at first and then more slowly as the reaction progressed   When the specific activity reached approximately one-half that of the original pepsin there was no further appreciable change with continued exposure to ketene   It was first thought that this flattening out of the

specific activity time curve indicated the completion of the acetylation reaction and a product of definite properties different from those of pepsin was repeatedly obtained The pH, however, was not held constant The acidity of the enzyme solution changed from pH 5 5 to 4 0 during the course of the acetylation experiment This might be expected for, as previously pointed out, ketene reacts with water to form acetic acid

When the acetylated product described above, with a specific activity of 60 per cent that of pepsin, was precipitated out of the pH 4 0 buffer and dissolved in pH 5 5 acetate buffer and ketene run in as before, the specific activity again dropped and again reached a nearly constant value as the pH of the solution approached pH 4 0 This product was precipitated and redissolved in fresh pH 5 5 buffer and as before subjected to ketene and again there was an initial drop in activity followed by a slowing up of the time rate as the pH of the solution dropped toward pH 4 0

*Experiment I—Course of Acetylation as pH Varied from pH 6 0-4 0 —*175 gm of twice crystallized Cudaby pepsin filter cake was dissolved in 3 M sodium acetate, final volume was 400 ml Ketene was run in with stirring 20 ml samples were withdrawn at various intervals of time and the following analyses made using the methods described in the section devoted to experimental methods—pH, protein nitrogen, non protein nitrogen, Van Slyke amino nitrogen, proteolytic activity by the hemoglobin method, and acetyl content After 800 minutes exposure to ketene the protein was precipitated out by the addition of an equal volume of saturated magnesium sulfate The precipitate was filtered off, dissolved in 3 M sodium acetate, and the final volume made up with sodium acetate to the volume of the solution just prior to precipitation Ketene was passed in as before for 900 minutes or a total of 1700 minutes The protein was again precipitated as before and dissolved in fresh sodium acetate and ketene run in for 300 minutes or a total of 2,000 minutes The analyses of the samples are plotted in Curve I of Fig 2, Curve I of Fig 3 and in Fig 5

The results of this series of experiments may be seen graphically in Curve I (Fig 2) They indicate that the change in slope of the time specific activity curve was due to a change in the pH of the solution and not to the completion of the acetylation It was later found that the pH could be kept nearly constant by placing the buffered pepsin solution in a collodion bag suspended in a large volume of buffer As a check experiment, therefore, acetylation was carried on in a

collodion dialyzing bag with the pH nearly constant between pH 5 0 and 6 0 Under these conditions the drop in specific activity of the reaction mixture with time did not show a decided change as the specific activity approached 60 per cent of the original pepsin As may be seen from Curve II of Fig 2 the activity continued to drop with only a very gradual change in the slope of the curve and seems to show that the differences noted in the acetylation of pepsin are differences in time rates and are probably caused by the differences noted in the pH values of the solutions However, when the increase in acetyl
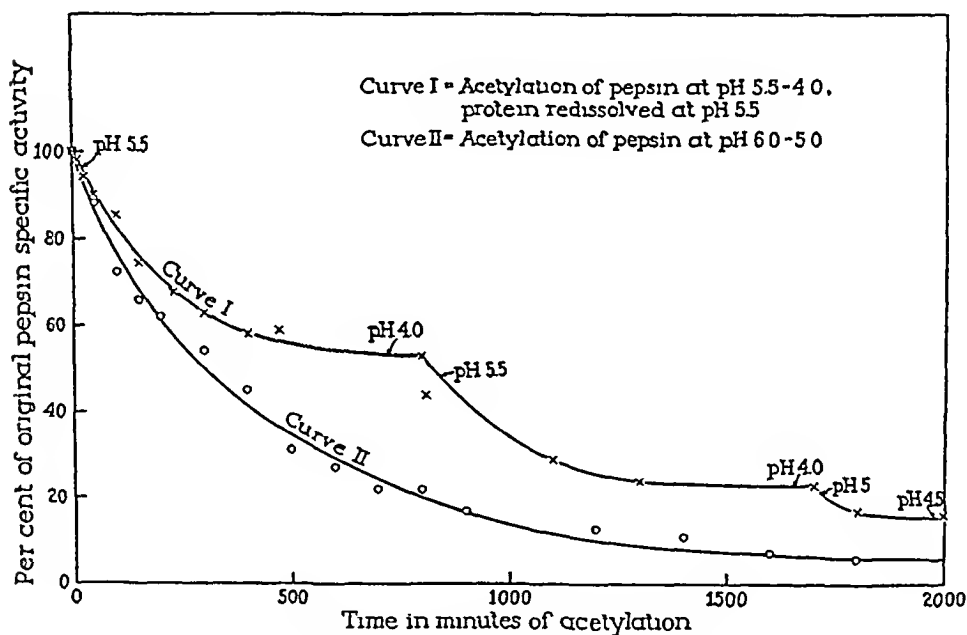
FIG 2 Per cent specific activity of pepsin during course of its acetylation

groups was plotted against specific activity (see Fig 3) the points obtained from acetylation at pH 4 5 and at pH 5 5 did not fall along the same curve

Experiment II—Course of Acetylation at Constant pH 5 5 —135 gm of once crystallized pepsin, prepared from Cudahy pepsin was twice precipitated by half saturated magnesium sulfate at pH 4 0 and dissolved in 2 M acetate buffer pH 5 8 so that the final volume was 400 ml The enzyme solution was placed in a collodion bag prepared from Merck's U S P collodion and this set into 2 liters of the same buffer as that used for the solution of the protein Ketene was run in rapidly and the solution stirred both inside and outside of the bag At the end of

the day the bag and solutions were placed in the ice box and the reaction started again the next morning   20 ml samples were withdrawn and analyzed for protein, non protein nitrogen, Van Slyke amino nitrogen pH, activity by the hemoglobin method, and acetyl content   The results of the analyses are to be found plotted in Curve II of Figs 2 and 3, Curve I of Fig 4, and in Fig 5

These curves are independent of time and should, therefore, be collinear if the time rate is the only factor in the acetylation of pepsin affected by a change in pH   There was a much greater drop in specific
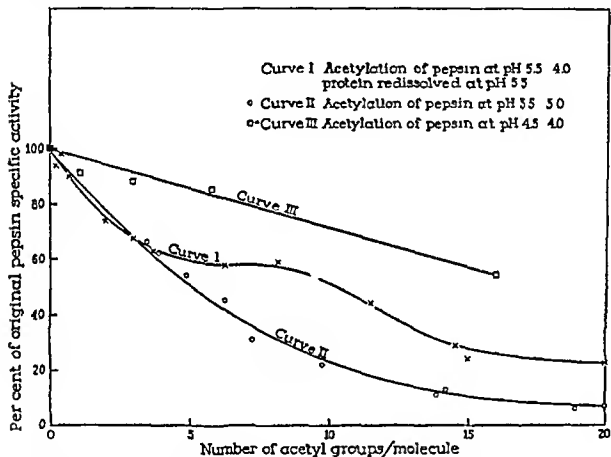
FIG 3 Effect of a change in acetyl groups per molecule on specific activity of pepsin

activity per unit increase in acetyl groups when the acetylation was carried on at pH 5 5 than at pH 4 5   Curves I and II of Fig 3 were plotted from data of the same experiments as were the Curves I and II of Fig 2   Curve III of Fig 3 represents an experiment in which the pepsin was acetylated at pH 4 5–4 0

Curves I and II of Fig 4 are the data of Curves II and III of Fig 3 plotted on logarithmic scale   It may be seen from Fig 4 that when the pH is constant the change in specific activity of pepsin during

acetylation varies logarithmically with the increase in acetyl groups per molecule

Experiment III—Course of Acetylation at Constant pH 4 5—45 gm of twice crystallized Cudahy pepsin filter cake washed four times with half saturated magnesium sulfate was dissolved in 6 M pH 4 5 acetate buffer so that the final volume was 150 ml   Ketene was passed in rapidly with stirring   20 ml samples were withdrawn and analyzed for protein, non-protein, and amino nitrogen, activity by the hemoglobin method, pH, and acetyl content

The results are plotted in Curve III of Fig 3, Curve II of Fig 4, and in Fig 5
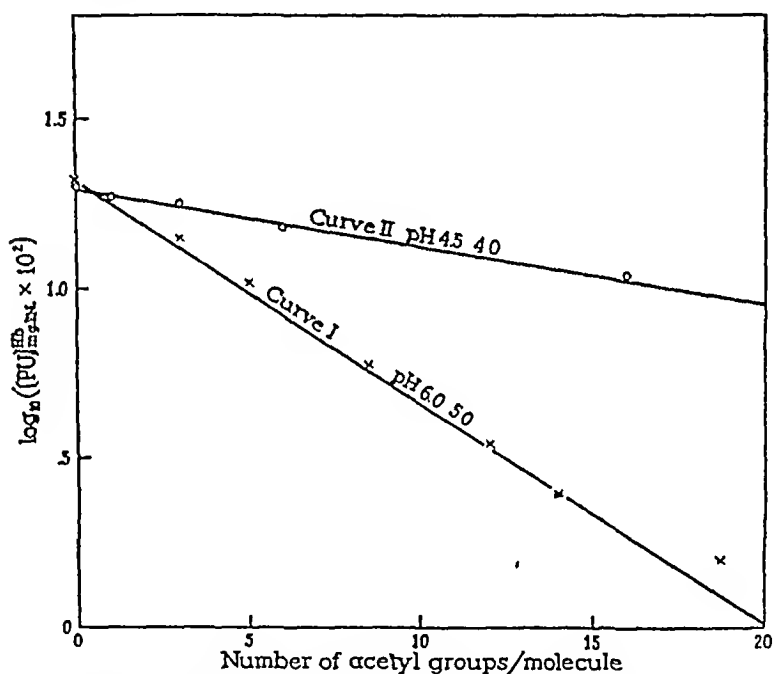
FIG 4 Logarithm of specific activity of pepsin as affected by a change in the acetyl groups per molecule, acetylation at nearly constant pH

It may be seen that there was only one-third the drop in specific activity per unit increase in acetyl groups at pH 4 5 as when the enzyme was acetylated at pH 5 5   Evidently acetylation occurs at different groups, depending upon the pH   This is probably due to the effect of ionization upon the rate of acetylation of the various groups present in the original pepsin

Change in Primary Amino Groups during Acetylation —The change in free primary amino groups was also followed during the course of the

acetylation reaction by means of Van Slyke's micro amino nitrogen method (15)

Pepsin contains an unusually small number of free amino groups, 0 15 per cent or three to four groups per molecule[1] calculated from a molecular weight of 36,000   Most proteins contain between 0 75–1 5 per cent amino nitrogen or twenty to forty free amino groups per molecule   Fig 5 contains the curves obtained by plotting the amino nitrogen content against specific activity as acetylation proceeds at pH 5 0–6 0 and pH 4 0–4 5   There seems to be no difference in the
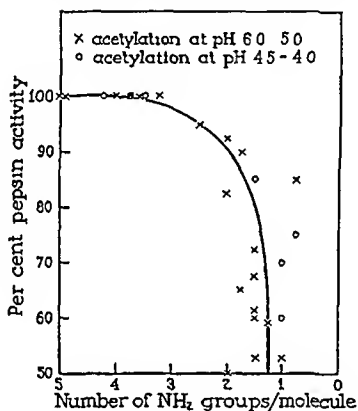
FIG 5 Effect of acetylation of free amino groups on specific activity of pepsin

loss in activity compared to the decrease in amino nitrogen whether the acetylation is carried out at pH 4 5 or pH 5 5   The results show that there is not more than a 20 per cent drop in specific activity with a loss of practically all of the amino nitrogen   Measurements of less than one amino group per molecule were unreliable

[1] This is lower than the value first reported (2) the difference probably being due to the presence of non protein nitrogen in the pepsin preparation of the earlier analysis

Preparation and Crystallization of the Acetyl Derivatives

Three preparations of acetylated pepsin have been obtained which differ in specific activities, number of acetyl groups per molecule, and in other properties For convenience they have been named according to their specific activity relative to that of pepsin and spoken of as though they were pure chemical individuals although in fact they may be solid solutions of closely related compounds

TABLE I

Preparations of Acetylated Pepsin

Preparation	Method of preparation	Specific activity $[P\,U]\frac{Hb}{mg\,P\,N}$	Pepsin specific activity	Amino nitrogen	No. of amino groups per molecule	Acetyl	No. of acetyl groups per molecule
			per cent	*per cent*		*per cent*	
Pepsin		0 22		0 16	4 0	0 12	1
"100 per cent active" acetyl pepsin	Acetylation of pepsin at pH 5 5 for 45 min	0 21	100	0 02	0 5	0 51	4
"100 per cent active" acetyl pepsin	Hydrolysis of "60 per cent active" acetyl pepsin in N/1 sulfuric acid at 8°C for 67 hrs	0 20	100	0 02	0 5	0 44	4
"60 per cent active" acetyl pepsin	Acetylation of pepsin at pH 5 5–4 0 for 12 hrs	0 12	55	0 04	1 0	1 0	8
"10 per cent active" acetyl pepsin	Acetylation of pepsin at pH 5 5 for 35 hrs	0 03	14			2 7	23

Experimental Procedure

"100 Per Cent Active" Acetyl Pepsin —This material was prepared by dissolving 100 gm of twice crystallized pepsin filter cake containing less than 10 per cent of the total nitrogen as non-protein nitrogen, in 300 ml of 3 M sodium acetate and then passing ketene into this solution slowly for 50–75 minutes One bubble of gas every second from an orifice 25 mm in diameter was found a satisfactory rate After 50–75 minutes analysis of protein, non-protein, and amino nitrogen, activity, and acetyl content showed a loss of not more than 10 per cent of the activity but a loss of all measurable amino nitrogen The protein was precipitated from solution by the addition of an equal volume of saturated magnesium sulfate after first adding acid to make the solution approximately pH 4 5 This precipitate was dissolved in water, precipitated by titrating to pH 3 0 with acid, and the precipitate

dissolved in water with the aid of sodium acetate and crystallized after the procedure of Northrop (2)

"*60 Per Cent Active*" *Acetyl Pepsin* —125 gm of once crystallized pepsin filter cake was dissolved in 3 M sodium acetate The final volume was 400 ml Ketene was passed in rapidly for 12–14 hours Analyses for protein non protein, and amino nitrogen, activity, pH, and acetyl content were made The solution was then subjected to the same treatment as the preceding solution, the protein precipitated with magnesium sulfate acid, and thence crystallized

' *10 Per Cent Active*" *Acetyl Pepsin* —125 gm of twice crystallized Cudahy pepsin filter cake was dissolved in saturated sodium acetate solution so that the final volume was 400 ml This solution was placed in a collodion bag of 500 ml volume The collodion bag containing the enzyme solution was placed in 2 liters of 3 M sodium acetate and ketene passed in for 35 hours The solution was filtered, and the protein precipitated and crystallized as above

Analytical results found in Table I are typical of several preparations of each product The derivatives were crystallized and were, in general, more stable than pepsin, possibly because they were less soluble than pepsin

The experimental procedure for the preparation of "100 per cent active" acetyl pepsin by acid hydrolysis of the "60 per cent active" acetyl pepsin is discussed on page 52

Evidence Concerning the Purity of the Crystalline Acetyl Derivatives of Pepsin

Fractionation Experiments —No evidence could be found for the existence of an acetyl derivative whose specific activity was between 100 per cent and 60 per cent of the activity of pepsin This follows from the results of the fractionation experiment summarized in Table II A large portion of a reaction mixture, the specific activity of which had been reduced to 75 per cent of its original value by acetylation with ketene, could be fractionated into two parts possessing approximately 60 per cent and 100 per cent of the specific activity of pepsin Further fractionation failed to demonstrate any protein fraction possessing a lower specific activity

It might be supposed that the "60 per cent active" acetyl pepsin was not a pure compound but a mixture or solid solution of pepsin and an inactive or weakly active acetyl derivative Table III contains the results of some experiments designed to test this question

The results in Part A are typical of several fractionation experiments on a number of the ten to fifteen preparations of "60 per cent active" acetyl pepsin The results in Part A show that the "60 per cent active" acetyl pepsin, as ordinarily prepared, could not be fractionated into pepsin and a protein having a lower specific activity A protein having an activity corresponding to that of pepsin was separated but the amount was insignificant though this amount of the material brings out the sensitivity of the fractionation experiments in detecting

TABLE II

Fractionation of a Reaction Mixture Possessing 75 Per Cent of the Original Activity

No	Volume	N/ml		[P U]^{Hb}		Total P.N	Total P.N
		Total	Protein	1/ml	1/mg P.N		
	ml	*mg*	*mg*			*mg*	*per cent*
No 4 of Dec 28, 1933, reaction mixture pH 6 0	1	20	14 2	13 1	2 1	0 16		
No 1 + 5 ml of 5 N sulfuric acid, stirred till precipitate dissolved, pH 4 5	2	25	11 4	10 5	1 7	0 16	263	100
No 2 + 7 ml saturated magnesium sulfate, filtered	3							
Residue No 3 dissolved in water	4	10	8 1	7 75	1 0	0 13	77 5	30
Filtrate No 3 + 9 ml saturated magnesium sulfate, filtered, filtrate	5	37	0 63	0 20	0 05	0 25	7 4	3
No 4 after samples had been removed	6	8	8 1	7 75	1 0	0 13	62 0	100
No 6 + 4 ml saturated magnesium sulfate, centrifuge, decant	7							
Residue No 7 + water	8	6		2 5	0 34	0 136	15 0	24

the presence of even closely related substances Part B of Table III reveals that an equimolecular mixture of pepsin and "10 per cent active" acetyl pepsin which resembled very closely the "60 per cent active" acetyl pepsin could be fractionated with comparative ease into two fractions having higher and lower activities than the mixture It seems probable, therefore, that the "60 per cent active" acetyl pepsin prepared according to the directions in the previous section is not a mixture or solid solution of the original pepsin with a derivative of very low activity

TABLE III

Fractionation of ' 60 Per Cent Active ' Acetyl Pepsin and of a Mixture of Pepsin and 10 Per Cent Active Acetyl Pepsin

		N/ml.		[P U]^{Hb}			
No	Volume	Total	Protein	1/ml	1/mg P.N	Total P.N	Total P N
		mg	mg			mg	per cent
Enzyme Solutions							
2 X crystallized Cudahy pepsin Nov 1 1933, dissolved in water + sodium acetate, concentration adjusted with water	1		5 65	5 50	1 13	0 21		
No 10 of Sept 25 1933 ' 10 per cent active" acetyl pepsin + saturated magnesium sulfate, filtered residue dissolved in water + sodium acetate	2		6 3	5 50	0 20	0 036		
No 5 of October 23, 1933, '60 per cent active acetyl pepsin	3		11 3	10 30	1 30	0 126		
Part A Fractionation of 60 Per Cent Active" Acetyl Pepsin							
No 3 + acid to pH 4 5 + equal volume saturated magnesium sulfate filter residue dissolved in M/10 pH 4 65 acetate buffer	4	75	7 14	6 9	0 84	0 122	520	100
No 4 + 22 ml saturated magnesium sulfate stirred centrifuge decant	5							
Residue No 5 + water	6	13	11 1	10 6	1 3	0 123	138	27
Supernatant No 5 + 40 ml saturated magnesium sulfate stirred (test showed practically no protein in solution) + 20 ml. M/10 pH 4 65 acetate buffer stirred 10 minutes and filtered filtrate	7	160	0 203	0 12	0 026	0 22	19	4
Part B Fractionation of an Equimolecular Mixture of Pepsin and 10 Per Cent Active Acetyl Pepsin							
10 ml No 1 + 10 ml No 2 (crystals formed 4 ml 4 N sodium acetate added to dissolve these crystals to neutralize the sodium acetate 3 ml of 5 N sulfuric acid was added 1 ml removed for analysis)	8	26		4 1	0 50	0 12	107	100
No 8 + 1 2 ml saturated magnesium sulfate with stirring centrifuge decant	9							
Residue No 9 dissolved in water	10	5		4 55	0 31	0 068	23	21
Supernatant No 9 + 10 ml. saturated magnesium sulfate centrifuge supernatant	11	40	0 69	0 36	0 088	0 24	14	13

Solubility Experiments

Experimental Procedure —The methods used in these solubility determinations were identical with those described by Northrop (16)

The solubility of a pure substance is independent of the quantity of solid phase   Northrop (2) found that in all the solvents used pepsin solubilities agree with this rule and the results indicate, therefore, that pepsin is a pure substance
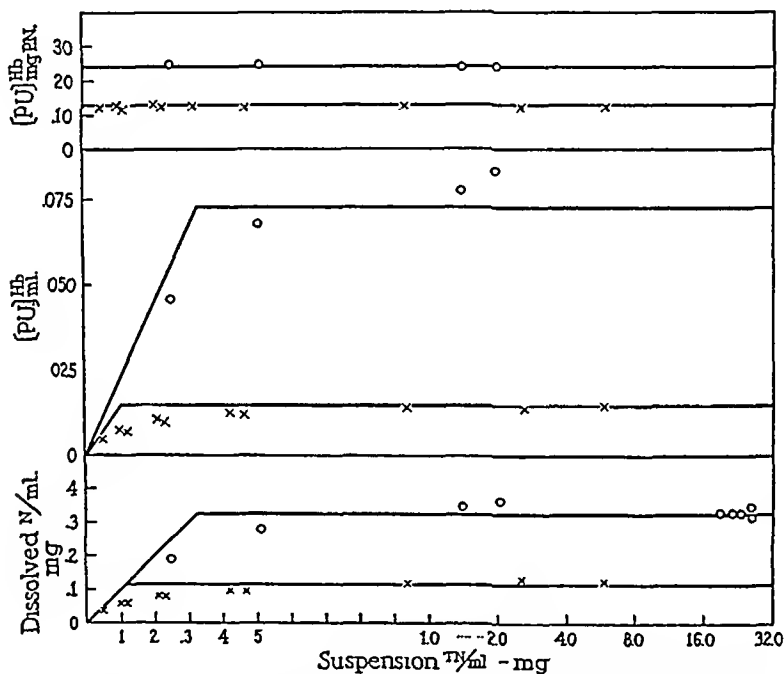
Fig 6 Solubility of amorphous "100 per cent active" acetyl pepsin and of amorphous "60 per cent active" acetyl pepsin in 0 05 M pH 4 65 acetate buffer and 0 5 saturated magnesium sulfate

The solubilities of two of the acetylated pepsin preparations have been determined   The results of these experiments are plotted in Fig 6   They are also summarized with the pepsin solubilities in Table IV   The results show definitely that the specific activity, *i e* ratio of activity to protein nitrogen, is constant and independent of the quantity of solid phase present   This confirms the results of the fractionation experiments   The points obtained in the region of a small quantity of solid material for the "60 per cent active" acetyl

pepsin do not fall on the theoretical curve   Although the figures are small and consequently subject to a greater percentage error, the writers believe that the results are significant in demonstrating that the material, as prepared, is a solid solution of two or more substances having nearly the same specific proteolytic activity

Solubility of Acetylated Pepsin in the Presence of Pepsin

Experimental Procedure —The suspensions of "60 per cent active" acetyl pepsin and of pepsin used in the solubility experiments of the previous section were used in the present experiments   The solvent was also the same (0 5 saturated magnesium sulfate—0 05 m pH 4 65 acetate buffer)   The two suspensions were mixed in varying quantities, keeping the final volume constant   They were then

TABLE IV

Solubilities of Pepsin and Acetyl Derivatives

Solvent— $\begin{cases} 0\ 5 \text{ saturated magnesium sulfate sp gr 1 295} \\ 0\ 05 \text{ m pH 4 65 acetate buffer} \end{cases}$

Temperature = 25°C ± 2 C

Material	N/ml.	
	Amorphous	Crystalline
	mg	*mg*
Pepsin (Northrop)	1 0	0 22
Pepsin (Herriott)	1 1	
100 per cent active acetyl pepsin	0 33	
60 per cent active acetyl pepsin	0 12	0 06

dissolved in the acetate buffer part of the solvent and precipitated by stirring in the saturated magnesium sulfate just as in the solubility experiments   In every case there was an excess of solid of both constituents after precipitation   They were then filtered and the filtrates analyzed for activity and nitrogen

The results of this experiment are shown in Fig 7   They bring out quite clearly that the "60 per cent active" acetyl pepsin forms solid solutions with pepsin

Constancy of Properties during Fractional Crystallization

Table V contains the analyses of seven fractional crystallizations of a preparation of "60 per cent active" acetyl pepsin   In the course of seven recrystallizations there is a loss of 80 per cent of the original material but no drift in any of the properties measured

Increase in Specific Activity on Removal of Acetyl Groups by Acid Hydrolysis

Since pepsin is instantly denatured in alkaline solution preliminary studies of the hydrolysis of acetyl groups from acetylated pepsin were
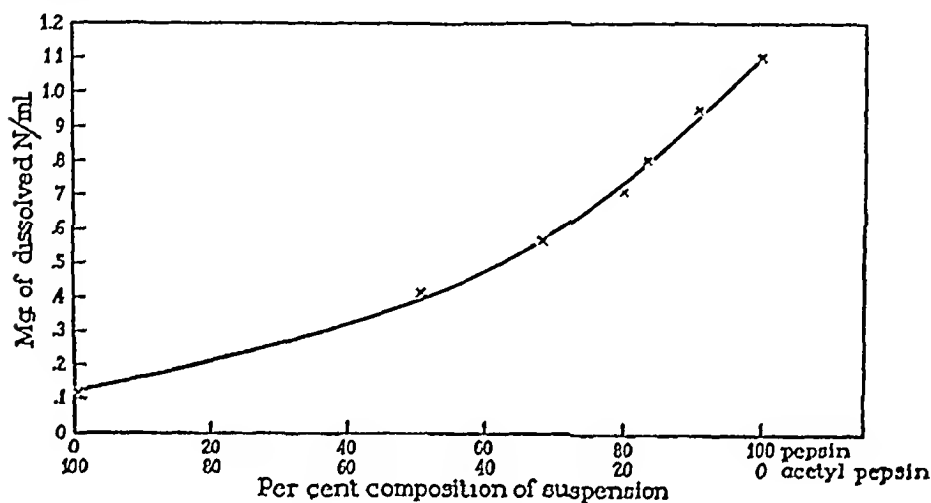
FIG 7 Solubility of mixtures of amorphous pepsin and amorphous "60 per cent active" acetyl pepsin in 0 05 M pII 4 65 acetate buffer and 0 5 saturated magnesium sulfate

TABLE V

Constancy of Properties of "60 Per Cent Active" Acetyl Pepsin during Fractional Crystallization

Crystallization No	Quantity of crystals	$[P\ U]_{mr}^{IIb}$ P N	$[\alpha]_D^{24°C}$ pII 4 7
	gm		
1	62	0 125	83
2	40	0 129	92
3	36	0 123	84
4	30	0 125	94
5	20	0 118	85
6	15	0 126	84
7	10	0 120	86

carried out in acid solutions At room temperature and with an acid concentration in which the hydrolysis of acetyl groups was appreciable there was too great a loss of enzyme through inactivation By

TABLE VI

Increase in Specific Activity on Removal of Acetyl Groups by Acid Hydrolysis

Procedure	No	Time in acid	Volume	N/ml		[P U]^{Hb}		NH$_2$-N No./mol	Acetyl No./mol
				Protein	Non protein	1/ml	1/mg P.N		
		h s	ml	mg	mg				
Acetylated pepsin No 15 of July 11 1933	1		130	12 8	0 5	1 15	0 090	0 -1	8-9
No 1 diluted with water to 2000 ml cooled to 10 C and poured into 2000 ml of cold 2 5 N sulfuric acid with mechanical stirring pH (electrometric by hydrogen electrode) = 0 02 A light precipitate formed	2	0	4000	0 416	0 016	0 0374	0 090		
5 ml suspension No 2 + 5 ml 1 3 N sodium acetate	3	0 03	8000	0 19	0 02	0 020	0 11		
5 ml suspension No 2 + 5 ml 1 3 N sodium acetate	4	2				0 0228	0 12		
5 ml suspension No 2 + 5 ml 1 3 N sodium acetate.	5	19		0 19	0 02	0 0272	0 14		
Aliquot of suspension filtered	6								
5 ml filtrate No 6 + 5 ml 1 3 N sodium acetate	7	19		0 044	0 036	0 010	0 23		
5 ml suspension + 5 ml 1 3 N sodium acetate	8	43		0 18	0 03	0 030	0 17		
5 ml filtrate + 5 ml 1 3 N sodium acetate	9	43		0 05	0 03	0 0108	0 22		
5 ml suspension + 5 ml 1 3 N sodium acetate	10	67		0 18	0 03	0 030	0 17		
5 ml filtrate + 5 ml 1 3 N sodium acetate	11	67		0 04	0 036	0 0115	0 29		
Suspension filtered after 67 hours in acid	12								
Filtrate No 12 + 950 ml saturated sodium acetate pH 2.5 + 2000 ml saturated magnesium sulfate filter Residue dissolved in acetate buffer	13			9 5	1 9	1 9	0 20	0 -1	3-4
$[\alpha]^{23 \ C}_{D \ pH \ 4 \ 5} = 90$ Crystals were indistinguishable under the microscope from pepsin									
Residue No 12 dissolved in water + sodium acetate pH 3 8	14		800						

TABLE VI—*Concluded*

Procedure	No	Time in acid	Volume	N/ml Protein	N/ml Non protein	[P U]Hb 1/ml	[P U]Hb 1/mg P-N	NH₂-N No /mol	Acetyl No /mol
		hrs	ml	mg	mg				
No 14 cooled to 10°C and added to 800 ml cold 2 5 N sulfuric acid, a light precipitate formed	15		1600						
5 ml suspension No 15 + 5 ml 1 3 N sodium acetate	16			0 270	0 018	0 048	0 18		
5 ml suspension + 5 ml 1 3 N sodium acetate	17	16		0 28	0 012	0 048	0 17		
Aliquot of suspension filtered	18								
5 ml filtrate No 18 + 5 ml 1 3 N sodium acetate	19	115		0 038	0 018	0 0091	0 24		
5 ml suspension + 5 ml 1 3 N sodium acetate	20	115		0 110	0 020	0 0216	0 20		
5 ml suspension + 5 ml 1 3 N sodium acetate	21	210		0 099	0 03	0 021	0 21		
Suspension + 550 gm sodium acetate, saturated with crystalline magnesium sulfate, filtered	22								
Residue No 22 dissolved in acetate buffer, + equal volume of saturated magnesium sulfate, filtered Residue = 15 gm, dissolved in water + acetate buffer, pH 4 5	23		25	17 7	0 8	3 5	0 20	2	2

carrying on the hydrolysis at 10°C there was practically no loss of protein in 10 days   Table VI contains the description and results of an experiment on the acid hydrolysis of an acetylated pepsin preparation The results show that with the removal of acetyl groups there was an increase in specific activity   The specific activity rose to that of pepsin where it remained practically constant   The acetyl content of the protein changed from eight or nine acetyl groups to three or four   Since there were still no free amino groups it is probable that these three or four acetyl groups are on the primary amino groups of pepsin and that this compound, therefore, corresponds to the "100 per cent active" acetyl pepsin prepared directly by slow acetylation of pepsin   On subjecting this material to a much longer treatment

with acid a substance was obtained which has the same specific activity but now has two free amino groups and only two acetyl groups per molecule

It is interesting to note that in general hydrolysis of acetyl groups linked to nitrogen atoms ($R - NH - C - CH_3$) requires very strong

$$\overset{\|}{O}$$

acid or alkali and high temperatures, whereas acetyl groups linked to oxygen ($R - O - C - CH_3$) are hydrolyzed with comparative ease

$$\overset{\|}{O}$$

TABLE VII

Specific Activity of "60 Per Cent Active" Acetylated Pepsin by Different Methods

Substrate	Method	Crystalline pepsin $[P U]_{mg\ P.N}$	Crystalline 60 per cent active acetyl pepsin $[P U]_{mg\ P.N}$
Hemoglobin	Colorimetric estimation of liberated tyrosine	0 23	0 13
Casein	Viscosity (increase)	310	190
	Viscosity (decrease)	1700	850
	Solution	0 55	0 25
Edestin	Viscosity (decrease)	2200	1100
	Formol	7×10^{-3}	4×10^{-3}
Gelatin	Viscosity (decrease)	24	17
Rennet	Viscosity (increase)	33×10^4	23×10^4

General Properties

Activity by Various Methods —The specific activity ($[P U]_{mg\ P.N}$) of a preparation of "60 per cent active" acetyl pepsin was determined by several different methods as described by Northrop (17) Although the specific activity of pepsin has been determined previously, using these same methods, the measurements are not all precisely reproducible (owing to variations in the substrate preparations) so that the activity of a standard pepsin solution was measured at the same time The results given in Table VII show, within the limits of the methods,

that the "60 per cent active" acetyl pepsin is a little over half as active as pepsin, by all of the methods tried

pH Activity Curves —It was thought that the shape of the pH activity curve of pepsin might be altered when the enzyme was acetylated. The pH activity curves of "60 per cent active" acetyl pepsin and of pepsin were determined by three different methods. The results in Fig 8 show that the shape of the pH-activity curve of pepsin is not changed by acetylation of pepsin

Experimental Procedure

1 Enzyme Concentration —The enzyme solutions were diluted for each measurement so that the hydrolysis of the protein was carried to the same extent in each case. The activity was calculated as the reciprocal of the concentration of enzyme required to cause the same rate of hydrolysis at the various pH and is expressed as per cent of the maximum activity

2 Substrates —Aliquots of dissolved Hoffmann La Roche crystalline edestin and dialyzed Ox carbon monoxide hemoglobin solutions were added to equal volumes of hydrochloric acid of different concentrations. The pH of the solutions were determined electrometrically by a hydrogen electrode. The final concentration of edestin was 3 per cent and of hemoglobin was 2 per cent

3 Methods—

(a) Hemoglobin

　　1 Phenol-colorimetric, see Anson and Mirsky (18)

　　2 Non-protein nitrogen. 1 ml of enzyme solution was added to 5 ml of hemoglobin in a 50 ml test tube. After 5 minutes the reaction was stopped by adding 10 ml of M/5 pH 7 3 phosphate buffer from another 50 ml test tube. The solution was mixed quickly by pouring back and forth from one tube to the other and then heated to boiling for 1 minute after which it was cooled quickly. This suspension was filtered through a Whatman No 42 filter paper and refiltered if the filtrate was not perfectly clear. 5 ml of the filtrate was analyzed by the Kjeldahl method for total nitrogen (19)

(b) Edestin

　　1 Non-protein nitrogen. 20 ml of 3 per cent edestin solution and 5 ml of enzyme solution were brought to 35 5°C in two 50 ml test tubes and then mixed by pouring from one test tube to the other. After mixing, 5 ml of the mixture was pipetted into each of three 50 ml test tubes. The hydrolysis in the tubes was stopped after 1, 5, and 10 minutes by the addition and subsequent mixing of 5 ml of 0 1 saturated sodium sulfate solution. After allowing these solutions to stand 20 minutes they were filtered through Whatman's No 42 filter paper and 5 ml of the filtrate analyzed for total nitrogen. The results of the three determinations at each pH were plotted, the slope determined and then corrected for the enzyme concentration. These corrected figures are plotted in Fig 7 as per cent maximum activity against the pH

Isoelectric point —Since the primary amino groups are altered in the acetylation of pepsin one might expect a difference between the isoelectric points of the acetylated products and the original pepsin Northrop (16) has found from minimal solubility and cataphoresis experiments that the isoelectric point of pepsin is close to pH 2 7 Microscopic examination (20) of an amorphous suspension of "60 per cent active" acetyl pepsin in hydrochloric acid of different concentra-

Pepsin by hemoglobin colorimetric method
× Pepsin by hemoglobin non-protein nitrogen method
60% active acetyl pepsin by hemoglobin colorimetric method
60% active acetyl pepsin by hemoglobin non-protein nitrogen method
▽ 60% active acetyl pepsin by edestin non-protein nitrogen method
▵ Pepsin by edestin non-protein nitrogen method
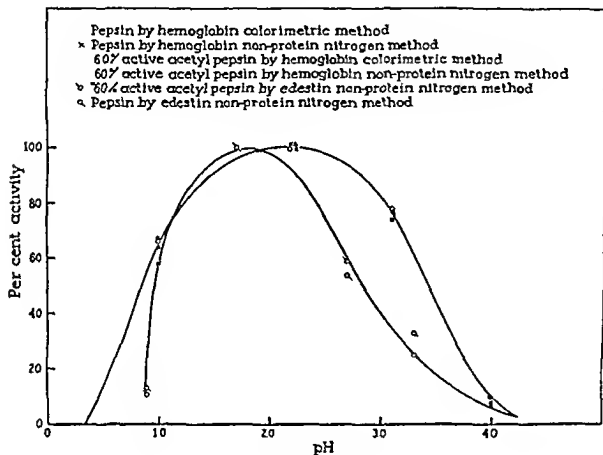
FIG 8 Percentage activity at various pH of pepsin and '60 per cent active" acetyl pepsin

tions revealed no change in the direction of migration   The particles of acetyl pepsin migrated to the anode at pH 3 0 and at pH 0 0 showing that within this range of pH the enzyme was negatively charged The minimal solubility was determined for the "60 per cent active" and for the "100 per cent active" acetyl pepsins   In these experiments a large excess of solid material was present   The results plotted in Fig 9 represent the solubilities of the amorphous materials in sulfuric acid solutions

Experimental Procedure

Enzyme Solutions —

(a) "100 per cent active" acetyl pepsin Dec 13, 1933, containing 8 7 mg PN/ml and less than 2 per cent non-protein nitrogen

(b) "60 per cent active" acetyl pepsin Dec 13, 1933, containing 16 mg PN/ml and 4 per cent non-protein nitrogen

Procedure —3 ml of enzyme solution was added to 10 ml of sulfuric acid of various concentrations at 23°C with rapid stirring in a 50 ml centrifuge cup After stirring for 1 minute they were centrifuged and the clear supernatant liquid
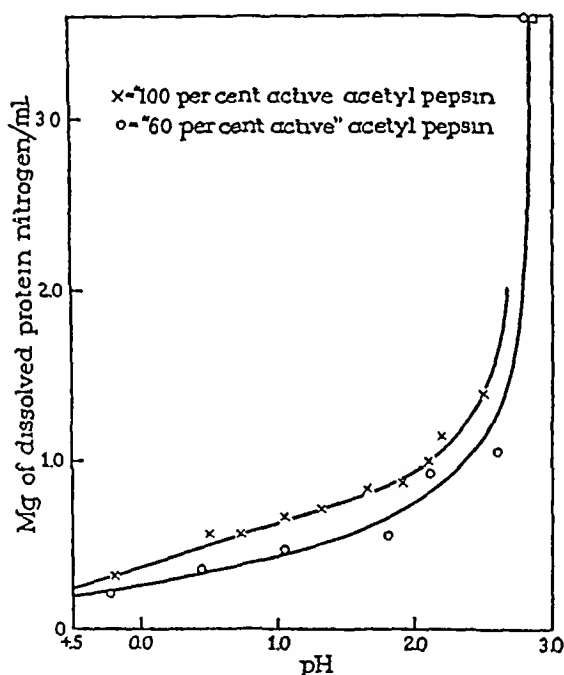
FIG 9 Solubility of amorphous "100 per cent active" and "60 per cent active" acetyl pepsin in various concentrations of sulfuric acid

analyzed for nitrogen, activity, and pH   Stirring for a longer time did not alter the solubility appreciably   The pH was determined electrometrically with a hydrogen electrode

The results in Fig 9 show no minimal solubility but a very gradual decrease in solubility as the pH decreases   These results confirm the cataphoresis experiments in showing that the acetylated products have no isoelectric point

Titration Curves —The titration curves of pepsin and three of the

acetylated products were determined, corrected, and are plotted in Fig 10 In titrating the "10 per cent active" acetyl pepsin, constant E M F readings could not be obtained in solutions more alkaline than pH 9 0 The drift was toward the acid region which seems to indicate that some hydrolysis takes place in solutions more alkaline than pH 9 0 and the hydrolysis products combine with the alkali Up to pH 9 0 the E M F readings were perfectly constant

It might be expected that acetylation of the amino groups in pepsin would result in a difference in the titration curves at approximately pH 8 0 where the free amino groups usually titrate This difference, however, of four amino groups per molecule is only 0 05 millimol of combined sodium hydroxide per gram of protein which could not be detected with any degree of certainty

Experimental Procedure

The crystal filter cakes of the different acetyl preparations were washed a number of times with half saturated magnesium sulfate and while on the funnel from the last filtration were washed with small quantities of dilute hydrochloric acid until the filtrates gave no sulfate precipitate on the addition of barium ion The hydro chloric acid was washed out with N/10 potassium chloride solution 10-15 gm of filter cake was then stirred to a paste and diluted to 50 ml with N/10 potassium chloride solution and the suspension placed in a cell connected to a calomel half cell by means of a saturated potassium chloride agar bridge The solution was stirred rapidly but did not foam 2 05 N sodium hydroxide was run into the suspension from a 5 ml buret through a long capillary extending under the surface of the solution A hydrogen electrode, standardized before and afterwards with N/10 hydrochloric acid was used in the determination of E M F When the solution reached pH 12 0 the volume and nitrogen content were determined.

Enzyme Preparations Used for Titration Curves 1 Pepsin—Twice crystallized pepsin No 1 of Nov 21, 1933, contained 10 per cent non protein nitrogen 4 8 gm of pure protein calculated from the nitrogen analysis was used

2 "100 Per Cent Active" Acetyl Pepsin—No 3 of Dec. 7, 1933, contained 4 per cent non protein nitrogen, 2 8 gm of pure protein was used

3 ' 60 Per Cent Active' Acetyl Pepsin—No 13 of June 12 1933 and recrystallized on Dec 10 1933, contained 3 per cent non protein nitrogen 3 5 gm of pure protein was used

4 "10 Per Cent Active" Acetyl Pepsin—Dec. 28 1933 contained 4 per cent non protein nitrogen, 4 3 gm of pure protein was used.

Acid and Alkali Inactivation—In studying the acid and alkali inactivation of acetylated pepsins it was hoped that some difference

would develop which would indicate a new characteristic property produced by the introduction of acetyl groups into the pepsin molecule   The results are graphically represented in Figs 11 and 12   In Fig 11 the points for pepsin were taken from Northrop (21)   The procedure in the experiment on the acid inactivation of acetylated pepsin was the same as that used with acid inactivation of pepsin (21) so that the results may be compared   The change in activity could
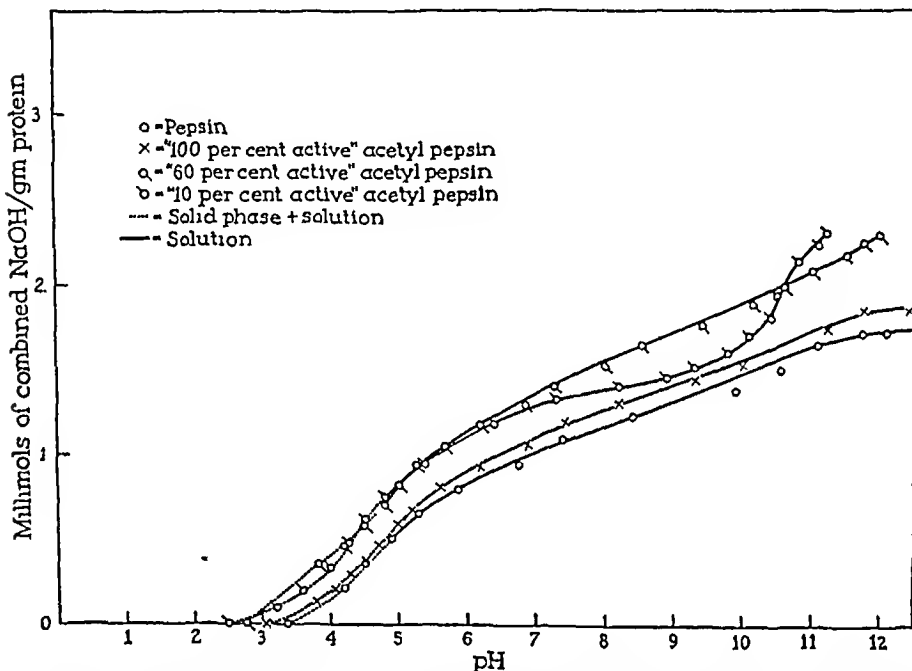
FIG 10   Titration curves of pepsin and three different acetyl derivatives of pepsin at 20°C

not be used as a measure of the loss in protein in the acid inactivation of acetylated pepsin for in strong acid the acetyl groups are hydrolyzed resulting in an increase in the specific activity of the protein

Experimental Procedure

1 Acid Inactivation —5 gm of once crystallized "60 per cent active" acetyl pepsin filter cake was dissolved in 250 ml of water with the aid of sodium acetate 5 ml of this solution was added to 20 ml of various concentrations of hydrochloric acid, the solutions were kept at 35°C and analyzed immediately and after 24 hours for protein nitrogen

2 Alkali Inactivation —The enzyme solutions were made up to contain 3 mg PN/ml 1 ml of this enzyme solution was then added to 5 ml of M/1 phosphate
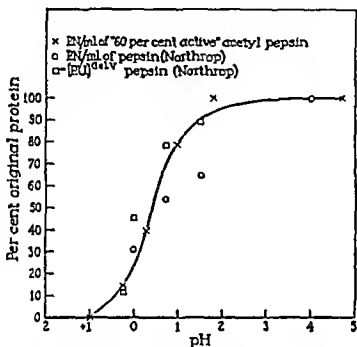
FIG 11 Per cent inactivation of pepsin and "60 per cent active" acetyl pepsin in various concentrations of hydrochloric acid at 35°C
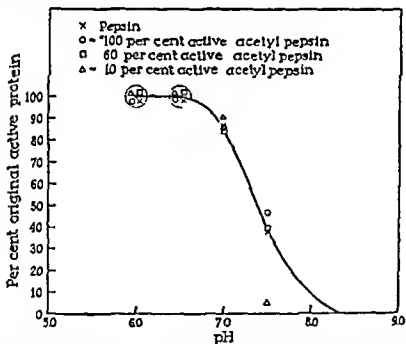
FIG 12 Per cent inactivation of pepsin and three different acetyl derivatives of pepsin in phosphate buffer at various pH and at 23°C

buffer at various pH and allowed to stand at 23°C After 5 minutes 5 ml of this solution was titrated with 5 ml of sulfuric acid of a concentration to bring the resulting solution to pH 6 0 1 ml was diluted for activity measurements by the

hemoglobin method, the dilutions being so arranged that approximately the same colorimeter reading was obtained

The results plotted in Figs 11 and 12 show that the acid and alkali inactivation of acetylated pepsin solutions follow approximately the same course as that of the parent protein, pepsin

One point might be mentioned which is not revealed in the figures and which, though not a difference in the proteins is of some interest A few moments after the acetylated pepsin was mixed with the 6 M hydrochloric acid a precipitate formed which remained during the 24 hours of the experiment   This precipitate was approximately 80 per cent of the original protein   It was completely inactive and insoluble under conditions in which the original material was active and soluble It was found to be denatured protein   Northrop (21) anticipated the presence of denatured pepsin protein in strong acid solutions but failed to find it   It appears that his solutions were not carried to concentrated enough acid for on similarly treating pepsin with 6 M hydrochloric acid the protein precipitated as denatured protein

EXPERIMENTAL METHODS

Acetylation with Ketene

The ketene used throughout this work was prepared by the thermal decomposition of acetone by means of a generator described by Herriott (14)   A 500 ml round bottom flask was employed to hold the acetone   A few alundum chips prevented superheating and bumping of the acetone as it was heated on a steam bath   A long water condenser followed by an ice trap removed most of the polymers of ketene and the escaped acetone vapor   The ketene was passed into the pepsin solution through an inverted 25 mm funnel   It was found that a 500 ml wide mouth Erlenmeyer flask was the most convenient container for the enzyme solution since it allowed the ketene to be introduced, the solution to be stirred with a mechanical stirrer, and samples to be removed without interference   During long (10 hour) exposures to ketene the Erlenmeyer flask was kept in water at 25°C ±2° for there is some heat developed on the introduction of ketene into the solution

pH Determinations

In general the pH estimations were made by the colorimeter method using the indicators recommended by Clark (22)

Activity Determinations

Unless otherwise specified the activity determinations throughout this paper were made by the colorimetric method of Anson and Mirsky (18)

Nitrogen Determinations

Protein nitrogen has been determined as the difference between the total nitrogen and the non protein nitrogen This is justifiable when the non protein nitrogen is a small part of the total nitrogen The enzyme solutions were diluted to contain 1–2 mg of nitrogen per ml, 1 ml was used for the total nitrogen, 2 ml was placed in a 50 ml centrifuge cup and 10 ml of boiling 5 per cent trichloroacetic acid added, the cup was shaken a moment and placed in a beaker of cold water After cooling the suspension was filtered and 6 ml of filtrate used for nitrogen analysis This is the non protein nitrogen All nitrogen determinations were made by the micro Kjeldahl method as described by Northrop and Kunitz (19)

Acetyl Estimation

A volume of enzyme solution containing 0 5–0 75 gm of protein was placed in a 150 ml beaker and this in turn placed in water at approximately 45°C At least five times that volume of boiling 5 per cent trichloracetic acid was added and the beaker placed immediately in ice water and the solution stirred rapidly This treatment precipitates the protein and cools the resulting suspension in a very short time After cooling the suspension was filtered without suction and the precipitate washed with distilled water until the filtrate was blue when tested with the indicator brom cresol green indicating that the filtrate was pH 5 0 or more This usually required from 15–20 washings with water The washing removed the acetic acid which had been present in the solution as acetate buffer but was converted to acetic acid by the excess trichloracetic acid The denatured protein is completely insoluble under these conditions When the precipitate had been washed free of acid most of the water was drawn off with the aid of suction and the precipitate dissolved in a small amount of dilute alkali or sodium phosphate After the precipitate had dissolved completely 1 ml of 4 N sodium hydroxide was added and 1 ml of solution withdrawn and analyzed for nitrogen from which the protein content was calculated The remaining liquid (approximately 11 ml) was put into a test tube and allowed to stand at 35°C for at least 5 days After this time 10 ml was put into a 150 ml modified Claissen distilling flask with 5 ml of 85 per cent phosphoric acid (reagent grade) and 0 2 ml of octyl alcohol The alcohol prevents foaming of the protein which would otherwise render vacuum distillation impossible The distilling flask was kept at 50–60°C on a water bath while the receiving flask was cooled with running water Distillation was carried out for 15 minutes at 20 mm of mercury pressure At the end of this time the receiving flask was removed the sides washed down with a few ml of water, a drop of 0 5 per cent

phenolphthalein added, and the solution titrated to a faint pink with N/50 sodium hydroxide   As a check on the titration figure and on the identity of the acid as acetic acid a drop of 0 1 per cent brom cresol green was added and the solution titrated back to pH 4 7 corresponding to the pK of acetic acid, with N/50 hydrochloric acid.   The second titration will be half of the first titration if only acetic acid is present in the distillate   After the titration the receiving flask was rinsed out with distilled water and fitted onto the distilling flask   5 ml of water and 0 2 ml. of octyl alcohol were added to the distilling flask and the distillation, followed by the titration, was carried out as before   This was repeated a third time which was usually sufficient to carry over nearly all of the acetic acid   A blank of 0 10 ml. of N/50 sodium hydroxide for each distillation and titration was deducted from
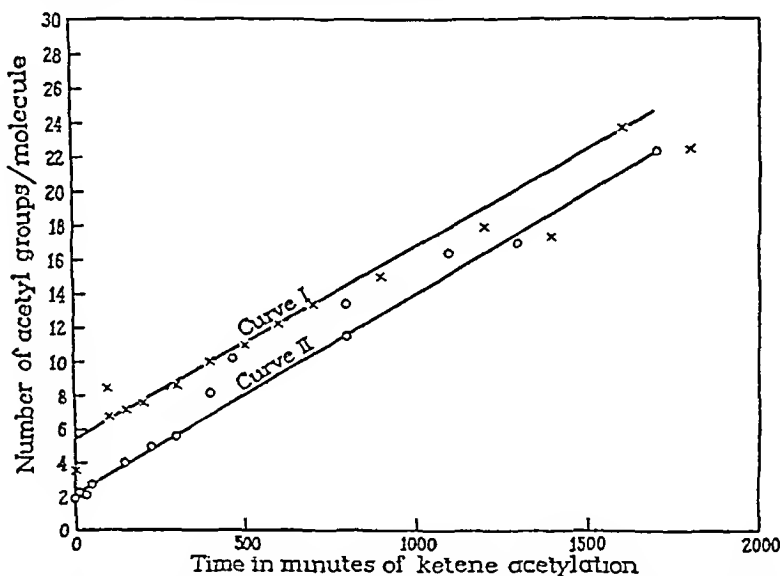
FIG 13   Change in acetyl groups per molecule of protein during acetylation

the total titration value before the amount of acetic acid distilled over was calculated

The above procedure was used for most of the acetyl estimations but in the case of pepsin a small amount of trichloracetic acid survived the washing and distilled over   This acid was probably combined with the free NH_2 groups as a salt   It was detected by the second or back titration to pH 4 7   When dilute sulfuric acid was used in place of trichloracetic acid this difficulty was avoided

To illustrate the precision of this method of acetyl estimation the increase in acetyl groups per molecule is plotted against the time of acetylation in Fig 13 Curves I and II were obtained from the same experiments as Curves I and II of Fig 3 were

Acetyl estimations were made on pepsin and acetylated pepsin by two methods other than the one described above   One of the two methods consisted in heating

a mixture of 5 ml of protein solution and 50 ml of a 25 per cent solution of p tolu enesulfonic acid to 110 C for 6 hours under a reflux condenser followed by the usual vacuum distillation and titration of the liberated acetic acid. In the other procedure the protein, in 0 4 N NaOH, was heated to 100 C under a reflux con denser for varying periods of time up to 5 days and the liberated acetic acid deter mined as in the other methods after acidifying the alkaline digest 2 ml of molar cadmium sulfate was introduced into the distilling flask to retain any hydrogen sulfide, which is liberated on acidification of the digest The results of the three methods agreed although by the last method a slightly higher acetyl figure was obtained for both pepsin and the acetylated pepsin but the difference agreed with the figures obtained by the other two methods

Amino Nitrogen

Pepsin decomposes rapidly into non protein split products which have a high amino nitrogen content and which can be removed only with difficulty The pepsin protein was therefore denatured, washed, and dissolved before analyzing for amino nitrogen It was denatured by adding five times its volume of boiling 5 per cent trichloracetic acid and the resulting suspension cooled quickly in cold water After washing the precipitate at least three times with several volumes of water or 2 5 per cent trichloracetic acid the residue was dissolved in dilute alkali or phosphate buffer The pH of the dissolved denatured protein was below pH 8 0 These solutions contained from 40-60 mg of protein per ml An aliquot of the solution was analyzed for total nitrogen from which the protein content was calculated The amino nitrogen was determined on the Van Slyke micro appa ratus (15) The nitrous acid protein mixtures were shaken for 20 minutes Most of the amino nitrogen estimations were made on solutions of the washed trichlor acetic acid precipitate These analyses were easily reproducible and agreed with the figures obtained by analysis of native pepsin which had been washed with half saturated magnesium sulfate and which contained less than 3 per cent non protein nitrogen The figures obtained from analyses of these trichloracetic acid pre cipitates were never higher than those obtained with native pepsin. This result shows that no hydrolysis of the protein occurs during denaturation unless there is a simultaneous coupling of an amino group at some other point The amino nitrogen content for native and denatured pepsin was also determined by Sören sen s formol titration as modified by Northrop (23) and found to agree with the values obtained by the Van Slyke nitrous acid method

SUMMARY

Crystalline pepsin has been acetylated by the action of ketene in aqueous solution at pH 4 0-5 5 As acetylation proceeds the activity decreases, the decrease being more rapid at pH 5 0-5 5 than at 4 0-4 5

Three acetyl derivatives have been isolated from the reaction

mixture and obtained in crystalline form   The crystal form of these derivatives is similar to that of pepsin

Fractionation and solubility determinations show that these preparations are not mixtures or solid solutions of the original pepsin with an inactive derivative

A compound which contains three or four acetyl groups and which has lost all of its original primary amino groups can be isolated after short acetylation   It has the same activity as the original pepsin A second derivative containing six to eleven acetyl groups has also been isolated   It has about 60 per cent of the activity of the original pepsin   A third derivative having twenty to thirty acetyl groups and about 10 per cent of the activity of original pepsin can be isolated after prolonged acetylation   The 60 per cent active derivative on standing in strong acid solution loses some of its acetyl groups and at the same time regains the activity of the original pepsin   The compound obtained in this way is probably the same as the completely active three acetyl derivative obtained by mild acetylation   These results show that acetylation of three or four of the primary amino groups of pepsin causes no change in the specific activity of the enzyme but that the introduction of acetyl groups in other parts of the molecule results in a marked loss in activity

The solubilities, amino nitrogen content, acetyl content, isoelectric point, and the specific activity have been determined by a variety of methods and found to be different from the corresponding properties of crystalline pepsin   The pH-activity curves, acid and alkali inactivation, and titration curves were not significantly different from the same respective properties of pepsin

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A CONVENIENT KETENE GENERATOR

By ROGER M HERRIOTT

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A simple apparatus for the preparation of ketene $(CH=C=O)$ from acetone vapor is described by Ott, Schroter, and Packendorff (1)   A more rigid and convenient apparatus has been designed and used by the writer (Fig 1)   Pyrex glass used in both the filament support and bulb reduces the possibility of cracking or breaking   The removable filament support makes it possible to replace the filament in a few moments   By turning up the ends of the 30 mill tungsten leads sealed into the glass, one may wind on the platinum or tungsten wire and thus rapidly replace the filament

Platinum wire (Brown and Sharpe gauge No 30) across a 110 volt circuit gives satisfactory results   A resistance in series with the lamp regulated to let 2 to 6 amperes through the filament results in a rapid evolution of ketene   After 8 to 12 hours continuous use there is a carbonaceous deposit on the filament   This deposit, being extremely brittle, may be removed by drawing the platinum filament over any sharp edge   The wire is then ready to be used again   A duplicate support and filament may be constructed which will be ready to replace the one in use if it burns out or breaks

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Ground glass joint

Filament support

Tungsten leads

to reflux condenser

Filament

0 1 2 3 4 5
Scale — cm

Acetone vapor

FIG 1

RESULTS OF IRRADIATING SACCHAROMYCES WITH MONOCHROMATIC ULTRA-VIOLET LIGHT

I Morphological and Respiratory Changes

By ROBERT H OSTER

(From the Laboratory of General Physiology, Harvard University Cambridge)

The development of quantitative methods for studying the effects of ultra violet radiation on bacteria by such workers as Ward (1893), Bang (1905), Bayne-Jones and von der Lingen (1923), and more recently by Gates (1929–30) and Wyckoff (1931–32), has only recently been applied to other organisms such as yeast Lacassagne (1930) exposed *S ellipsoideus* under a filter which transmitted the band of wave lengths of the mercury vapor arc spectrum between 2800 and 3800 Å u and noted that three categories, (*a*) immediate death, (*b*) deferred death (two cell groups), and (*c*) cells which, after a retarda tion in cell division, ultimately recovered their reproductive powers, could be distinguished in the irradiated cells

Wyckoff and Luyet (1931) showed that yeast exposed to high ener gies at several sharp bands in the mercury vapor spectrum fell into similar categories to those of Lacassagne, with the production of some giant cells Their results tended to indicate a multiple quantum hit relation between survival ratio and time of exposure

It is of interest to extend these results by a determination of the various factors involved in the selective absorption of radiant energy by the organism over a fairly complete range of wave lengths at care fully measured incident energies

The present investigation[1] was proposed by Professor W J Crozier as a search for stimulating and inhibitory effects of ultra violet energy

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upon small organisms   Yeast was chosen because of various obvious characters which commend its use for this purpose

This paper deals with the several reactions indicated by morphological changes, and with the respiratory activity of the yeast subsequent to irradiation   The influence of certain modifying factors, internal morphological changes, the lethal spectrum, and the relation between the survival ratio and the incident energy on the basis of quantum considerations, will be given later   The measured variables were   (1) wave-length, (2) intensity of the incident radiation, (3) period of exposure, (4) ratio of the number of cells forming normal colonies to the original number, (5) ratio of the number of cells surviving to the original number, (6) changes in cell size and structure, and (7) the number of cells formed per colony   In the respiratory changes the rate of consumption of oxygen by irradiated suspensions of yeast was studied in relation to the period of exposure

Methods

Light Source —The source of ultra-violet light was a vertical quartz mercury vapor arc with a tungsten anode, operated at 67 volts, 5 5 amperes, in series with a resistance from a 120 volt, D C supply line   It is practicable in the ultra-violet region between the wave-lengths 2225 and 3132 Å u to separate out lines, or groups of lines, of sufficient intensity at 2225, 2302, 2345, 2378, 2482, 2536, 2652, 2804, 2967, 3022, and 3132 Å u   The operating temperature of the lamp during the course of the experiment was controlled by a small blower set up to force a stream of air over the quartz tube

Quartz Monochromator —The characteristic light frequencies in the quartz region of the mercury arc spectrum were separated and focused on the test objects by a large quartz monochromator of special design   This was the same instrument used by Gates (1929–30 *a, b*, 1930–31) and assembled by him   A description of it is given in his article (Gates, 1929–30 *a*, p 233)

Energy Measurement —The energy incident upon the test object was measured in ergs per mm ^{2} per sec by a sensitive thermopile connected through a ballast resistance to a sensitive galvanometer (Gates, 1929–30 *a*, p 233)

Test Objects —All of the present experiments were made on *Saccharomyces cerevisiae* (Hansen), American Type Culture No 2335, originally obtained from Professor F W Tanner and maintained in this laboratory for a number of years by Dr A E Navez and Dr T J B Stier   This strain of yeast was chosen because of its long cultivation under standard conditions and because of certain known growth and temperature characteristics (Richards, 1928, and Stier, 1932–33) *S cerevisiae* being practically spherical does not offer serious orientation problems when seeded on an agar surface for irradiation   For most of the experiments

the yeast was transferred every 24 hours by inoculating a fresh slant of 2 per cent agar and 1 5 per cent malt extract (Difco) with a loopful of cells from the parent culture The nutrient medium on autoclaving for 20 minutes at 15 pounds pressure has a pH of 4 7 This is well on the acid side and is unfavorable for the rapid growth of most wild molds and bacteria encountered in the laboratory but favorable to the yeasts

EXPERIMENTAL

Suspensions of yeast of standard turbidity with a depth of disappearance of 70 mm (Gates, 1920) were made up from 24 hour cultures incubated at 25 C To break up any clumps of cells the suspensions were forced up and down 12 to 15 times through sterile cotton with a sterilized pipette On examination under the microscope such suspensions were found to contain less than 1 per cent of two-cell groups

The suspension was flowed over the surface of malt agar contained in small Petri plates 5 5 cm in diameter which were attached with wax to 5 1 × 7 6 cm glass slides The excess fluid was poured off and the plates allowed to stand in a vertical position until the water remaining on the agar surface had evaporated or had been absorbed

Inoculated plates thus freed of surface liquid, were covered with a crystal quartz plate to compensate for the quartz window of the thermopile and were mounted in a vertically placed mechanical stage in the plane of the thermopile junctions By a rack and pinion either the plate or thermopile could be brought into the path of the beam of light passed by the exit slit of the monochromator The seeded agar was irradiated over a sharply defined rectangular area of approximately 5 × 30 mm Four irradiated areas separated by equivalent control areas were obtained on each plate These were located at predetermined settings of the vernier scale of the mechanical stage The cells were irradiated with timed exposures and at measured intensities of the monochromatic radiation, at room temperatures between 22° and 25 C

After exposure the plates were incubated for 36 hours at 25 C [2] For examination under the microscope the exposed areas were located at corresponding settings on the vernier of a mechanical stage similar to that on the monochromator The central area of each exposed region between parallel ruled lines in the eye-piece and stops on the stage was searched in making each count Although intensity readings were taken before and after each set of exposures, and although the lamp was operated on high capacity storage batteries, certain experimental errors were found to occur These may involve uneven distribution of the incident energy over the area irradiated and possibly the partial protection occasionally afforded

Incubation temperature of 25 C was chosen because it is well below the critical temperature for this strain of yeast (30 C) above which the yeast although growing more vigorously gives rise to abnormal cells and colonies

by overlying cells, even after careful filtering and agitation of the inoculating suspension. Considerable variation in the distribution of the cells over the surface of the agar in the test plates may occur. These variations have been partially compensated by performing several similar experiments (4 to 7) at each wave-length studied, and by a statistical analysis of the data obtained. The number of cells in each irradiated area was found to vary between 150 and 300, so that for an average of five experiments a point on the final average smoothed curve at a given wave-length represents the counting of approximately 1000 cells. The percentage of cells growing into normal macroscopic colonies was determined and plotted against the incident energy as shown in Figs 1 and 2. To treat the data statistically, the method of averaging data from the smoothed curves of several parallel experiments was used as discussed by Gates (1929–30). A similar treatment was accorded the data obtained using the three-cell stage as a criterion of survival

Changes in Cell Size and Shape

In the earlier experiments, as in those with *S aureus* and *B coli* by Gates (1929–30), colony formation was chosen as a suitable all or none criterion of survival, no evidence of a stimulative action was found, even for exposures of 1 and 2 seconds. But observations with higher powers of the microscope revealed various changes and abnormalities[3] in cell growth and reproduction following irradiation, which indicated a period of survival even though normal colonies were not formed. On examination from 24 to 50 hours after irradiation, the extent of these changes was found to vary widely among the cells in a given irradiated area. This occurred at all of the wave-lengths studied between 2225 Å u and 3132 Å u

Similar to the findings of Lacassagne (1930) and Holweck (1930) different types of cell groups could be distinguished (1) single cells of normal size and showing no visible change, (2) single cells of giant size, (3) two-cell groups usually from 3 to 8 times the size of normal two-cell groups and more spherical in shape, but often, in the budded cell, showing a long filament-like process constricted at intervals along its length, (4) three- to eight-cell groups, usually giants, which reach this stage and cease budding or go on to form buds of normal size to the production of a small colony, (5) larger groups in which although retarded in the formation of a normal sized colony show little evidence of giant cells

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[3] Wyckoff and Luyet (1931) have called attention to some of these changes since the first observations were made in this laboratory. After the exposure of yeast from 15 day cultures they observed giant cells and cell colonies of reduced size which, although not reproducing, seemed to be "alive"

Inhibition of Reproduction

It is evident that two visual criteria can be selected for judging the effect of ultra violet radiation upon the yeast cell. The first of these, inhibition of reproduction, is arbitrarily defined as the checking of budding to such an extent that the cell cannot give rise to a normal sized macroscopic colony before the environmental conditions of nutrition and waste products inhibit further activity. Obviously, various stages of inhibition may occur.
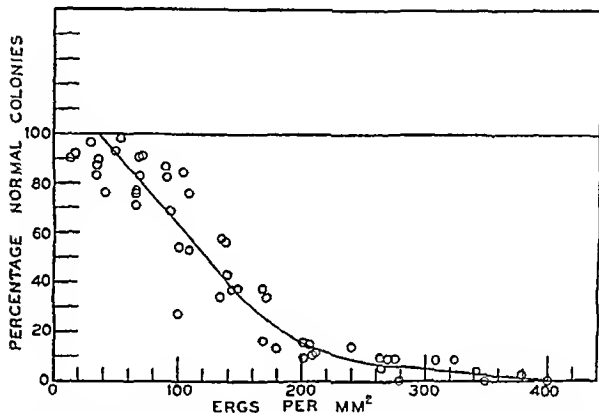
FIG 1 The course of the inhibition of normal colony formation at 2536 Å u The curve is drawn through the average points obtained from the smoothed curves of five experiments for comparison with points of single observations

Using this criterion, a definite, measurable, retardation could be demonstrated at the wave lengths 3022, 2967, 2804, 2652, 2536, 2482, 2378, and 2225 Å u. Areas irradiated at these frequencies showed that the size of the colonies decreased at a rate roughly proportional to the increase in incident energy. The relation between the number of normal sized colonies in the irradiated area as compared to the number of normal sized colonies in equal control areas on each side of the exposed area and the incident energy is shown in Fig 1, where the

smoothed curve is drawn through the points obtained from five similar experiments at this wave-length   In Fig 2 the curve is plotted and drawn from data obtained from averaging the values taken from the smoothed curves of each of these experiments   Points taken arbitrarily at intervals of 10 per cent (clear circles) from the curve of Fig 1 fall very close to the mean curve
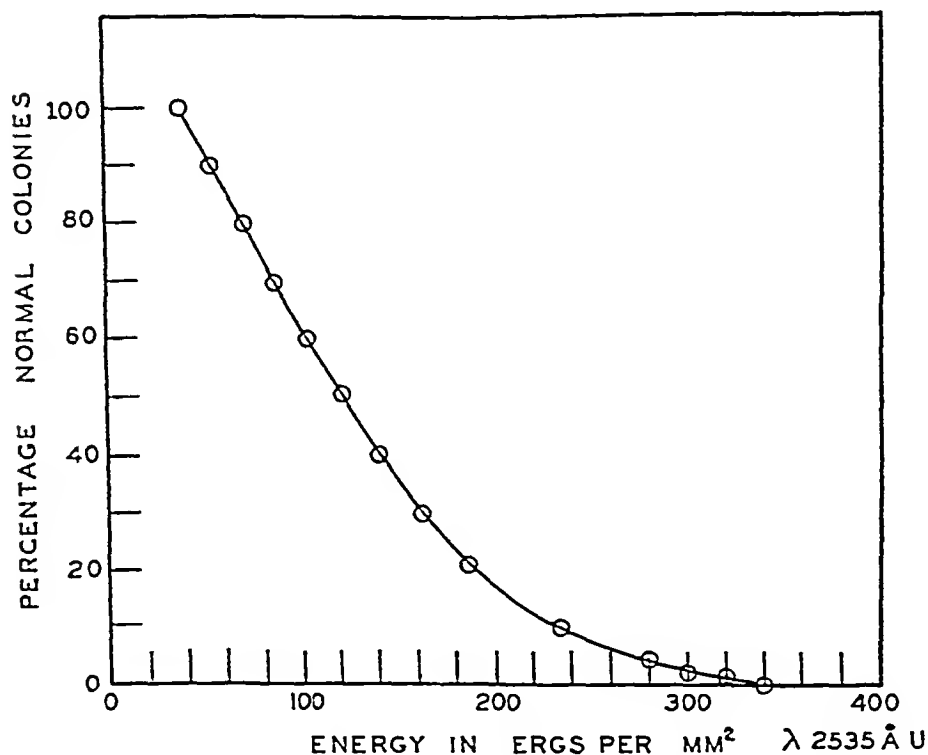
FIG 2 The course of inhibition of normal colony formation of yeast cells irradiated at 2536 Å u , plotted from mean values obtained from smoothed curves of five parallel experiments   Circles indicate averages of single observations of four experiments grouped at 10 per cent intervals

From these curves the relation between the percentage of inhibition and the incident energy appears to be logarithmic over the inhibitory range   There seems to be some resemblance to a first order reaction curve but the same argument holds here in the case of yeast as has been presented by Gates (1929–30), namely, that a distribution of resistance factors in the individual cells probably appreciably modifies

the shape of the curve from that of a true first order reaction This point will be referred to in a subsequent paper dealing with the quantum relations between light energy and the biological effect In a 24 hour culture budding is still occurring at a rapid rate (Fig 3), so there will be a mixed population of cells of different ages, and hence some variation in their reaction to absorbed quanta
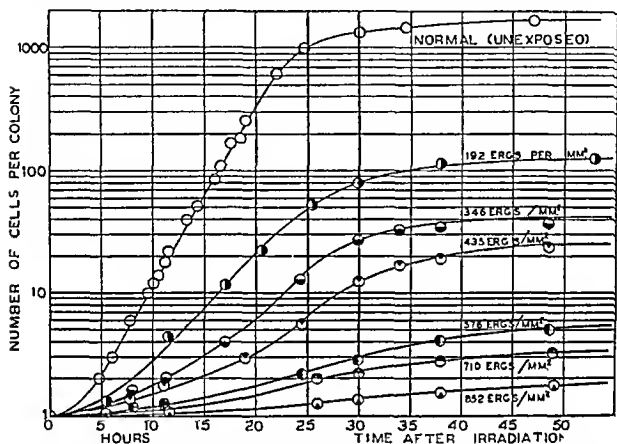
FIG 3 The effect of ultra violet light (2652 Å u) on the rate of reproduction of yeast. Curves showing the increase in the number of cells per colony in normal and irradiated areas

Cells exposed to the other lines of the mercury vapor arc spectrum showed the same type of curve but the energies required to effect the same degrees of inhibition differed with the wave length indicating that the absorption coefficient varies with the frequency of the incident radiation

Inhibition of reproduction, i e the inability to form normal sized colonies, constitutes a satisfactory and clear cut end point for what appears to be the primary effect of absorbed ultra violet energy It has been noted, however, that when the period of exposure is increased

beyond this point to higher energies varying degrees of injurious effects occur until all budding and growth are stopped   In analyzing this more extended part of the reaction a second end-point was chosen to serve as a criterion for measuring the survival of irradiated cells

Lethal Effect

Survival may be judged in various ways, such as the resistance of the cell to penetration by certain dyes (*cf* Fulmer and Buchanan, 1923–24, Fraser, 1920), the continuance of gas exchange, or fermentation, etc , but for this study the ability of the yeast cell to form two or more daughter cells was selected as the criterion of survival   The survival ratio (Swann and del Rosario, 1932) then becomes the ratio of the number of cells which bud at least twice to the original number of cells exposed [4]

Using this criterion, detailed counts of the groups developing from irradiated cells were made, after incubation in darkness for 36 hours at 25°C   The survival ratio (N/N_o) was calculated from the data of each experiment (after correcting for the number of apparently dead single cells as obtained from average values from the adjacent control areas) and the mean values were obtained by the method used in studying inhibition of colony formation for points arbitrarily taken at intervals of 10 per cent on the smoothed curves of several experiments at each of the wave-lengths studied, as given above

The protocol of a single typical experiment at 2652 Å u is given in Table I to show the different classes into which the groups of cells were placed   The

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[4] The use of the two-cell stage as an end-point appears to be unsuitable   The difficulty of preventing the presence of some two-cell groups in the original inoculating suspension, even after frequent filtering through cotton and great dilution with distilled water, and the possibility that a first budding may occur in a few cells in the interval between inoculation of the plate and its exposure, make the two-cell stage unsatisfactory as an index of survival   Again, when irradiated groups of cells are washed gently with eosin water stain at from 30 to 50 hours after irradiation, many of the two-cell groups are found to take up stain in one or both of the cells in a manner similar to the way cells killed by heat take up this stain   Also on examining the exposed areas at from 60 to 72 hours after irradiation, many of the two-cell groups have fuzzy indefinite edges, indicating that cytolysis has occurred

actual number of each class has been converted into percentage of survival after correcting for the number of single cells found in equal control areas   Curves were plotted from these data for the single cells, two-cell groups, and survivors (all groups of three or more cells)   By performing several experiments at each wavelength the ends of the survival curve can be determined with much more accuracy than by any single complete experiment   The average deviation was found to vary between 4 and 9 per cent at the lower end of the curve for all wave lengths and between 4 and 11 per cent at the upper end   In no case was the maximum per cent deviation twice that of the average deviation

TABLE I

Protocol of a single experiment carried out at wave length 2652 Å.u at 23° (Experiment 45) and examined after 36 hours

Energy	Single cells	2-cell stage	3-4 cell stage	5-16 cell stage	16-cell stage
ergs/mm [1]	*per cent*	*per cent*	*per cent*	*per cent*	*pe cent*
101	0	0	0	0	100
152	4 0	4 0	1 0	0	91
203	4 5	1 5	1 5	2 8	89 7
254	6 6	0	0	2 2	91 2
304	13 2	2 5	1 26	12 0	71 0
350	7 0	12 3	1 8	12 3	66 6
405	19 7	13 1	5 0	26 2	46 0
456	27 0	15 0	6 3	16 7	35 0
506	37 0	27 0	6 5	4 4	26 1
557	38 5	23 8	14 7	13 0	10 0
608	53 0	35 4	6 3	4 2	1 1
684	55 0	27 0	9 0	8 0	1 0
760	77 0	11 0	7 0	4 0	1 0
836	78 0	13 5	6 8	1 7	0 0
900	88 0	9 0	3 0	0 0	0 0
1000	93 5	5 0	1 5		
1150	96 5	1 5	2 0		
1217	99 0	0 0	1 0		
1268	100 0	0 0	0 0		

A summary of the mean values obtained by this method for the various representative lines of the mercury arc spectrum are given in Table II   In Fig 4 the curves for all wave lengths studied have been plotted with the exception of 3022 and 3132 Å u   Reference to Table II shows that the incident energy required to "kill" *S cerevisiae* at 3022 Å u is very much higher than at any of the shorter wave lengths studied   This fact was indicated in the study of inhibition   Expo

sures of an hour or more at 3132 Å u produced such slight effects in inhibiting colony formation that no further tests at this wave-length were performed

The curves of Fig 4 form a family of curves as judged by averaging the energies as well as the numbers of yeast cells in terms of 100 per cent   This gives a curve similar to that of any of the individual curves

Some energy is absorbed before any yeast cells react   For all wave-lengths included in Table II the energy incident upon the cells before

TABLE II

Summary of Survival of Saccharomyces cerevisiae after Irradiation with Mono-chromatic Ultra-Violet Light

Wave length, Å u	2225	2378	2482	2536	2652	2804	3022	
Percentage survival	Energy (Mean values)							
	ergs/mm	*ergs/mm²*	*ergs/mm²*	*ergs/mm²*	*ergs/mm²*	*ergs/mm²*	*ergs/mm²*	
0	1590	2980	1770	1290	1130	1820	63	10³
1	1540	2686	1580	1230	1070	1450	60	"
5	1340	2290	1353	1047	870	1157	54 2	"
10	1200	2003	1203	915	766	985	47 9	"
20	1018	1686	1042	751	652	818	39 6	"
30	895	1460	917	648	580	712	33 6	"
40	785	1314	803	566	518	630	28 5	"
50	677	1156	696	503	457	556	23 5	"
60	572	1030	593	440	398	489	19 6	"
70	472	880	500	378	343	419	15 0	"
80	363	720	395	311	282	360	11 0	"
90	258	556	292	240	220	275	7 2	"
100	169	320	180	155	131	191	4 5	"

any lethal effect is observed is approximately 8 per cent of that required to "kill" all of the cells   Between 20 and 30 per cent of the cells do not survive after an exposure relatively short when compared with that required to suppress budding of all of the cells   The remaining 70 or 80 per cent require from 70 to 80 per cent of the energy required to kill all of the cells   The last 2 or 3 per cent of the cells are affected at a slightly lower rate   The relation between survival and incident energy over the major portion of the lethal range is thus almost logarithmic

An analysis of the data obtained at the different wave lengths on the basis of the percentage of the different types of cell groups showed no significant differences in the relative proportions of the several types

Many two cell groups present after 24 hours incubation proceed to bud and form groups of three or more cells 36 hours after irradiation This point is illustrated by Fig 5, in which were plotted the percentages of the different cell groups in an area which received 554 ergs of energy per mm ² at 2652 Å u as determined after 24 and 144 hours
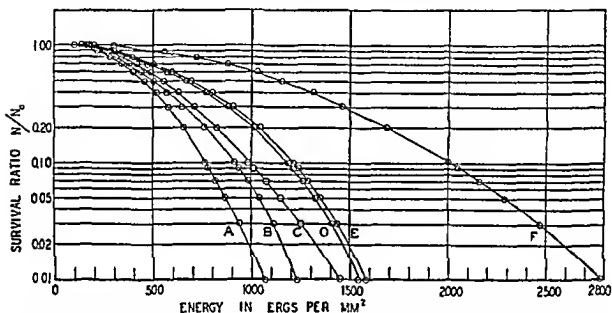
FIG 4 Survival of yeast cells irradiated with monochromatic ultra violet light Incident energies required for lethal action at various wave lengths   A, 2652 Å u , B, 2536 Å u , C, 2804 Å u , D 2225 Å u , E, 2482 Å u F, 2378 Å u

incubation at 25°C   Observations made over a period of 150 hours, on yeast incubated in a moist chamber showed that the average de crease in the number of two-cell groups (by budding) at from 36 to 150 hours was 5 per cent in an area exposed to a total incident energy of 1420 ergs per mm ² at 2536 Å u at 8°C and incubated at 25°C

The Shape of the Curve

The shape of the curves of Fig 4, especially through the middle region where the rate of killing is almost constant for each added increment of energy, resembles that of a first order reaction   Certain departures from the S curve of a true first order reaction indicate that other fac

tors beside absorbed energy probably play a part in influencing the course of injury   Lower resistance in young bud cells would explain the sharp curvature of the first part of the curve, high resistance of
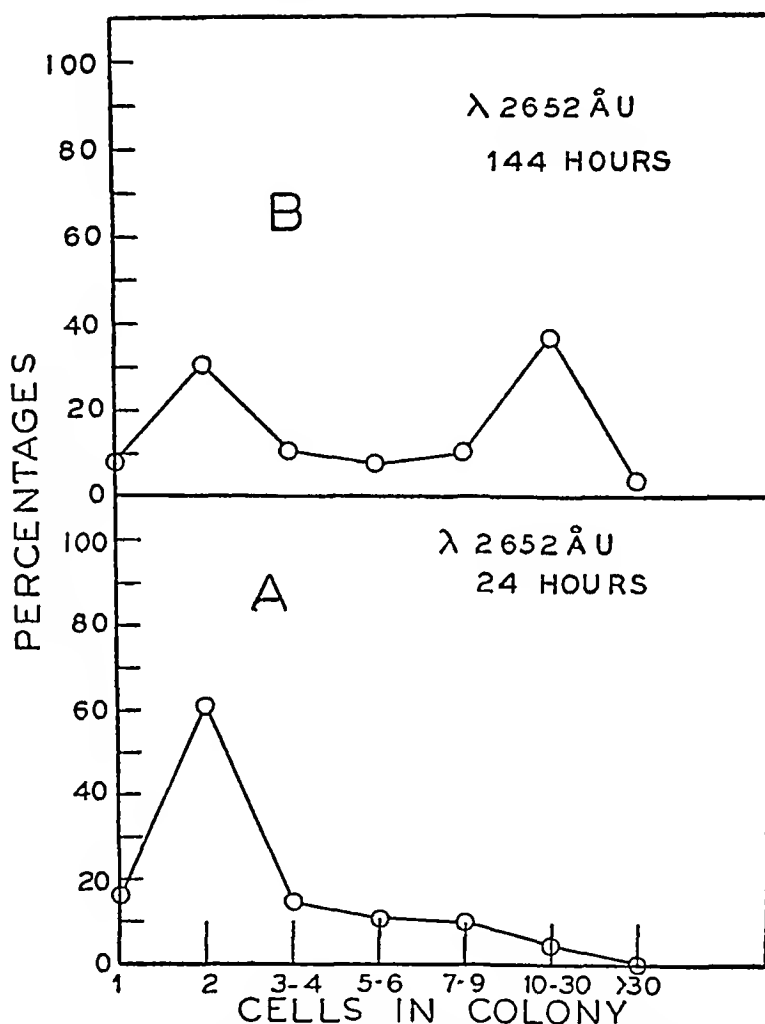
FIG 5 The relative numbers of different cell groups at (A) 24 hours after irradiation, and (B) 144 hours after irradiation, with an incident energy of 554 ergs per mm², and incubated at 25°C

old resting cells would tend to modify the shape of the lower end of the curve

If the influence of this factor is removed (assuming that the perfect state of all of the organisms being in the same stage of development

could be attained) we would expect a closer agreement between these curves and the theoretical curve based on the probability of absorbed quanta producing an effect, according to the hypothesis of Crowther (1924, 1926), Wyckoff (1930, 1931–32), Swann and del Rosano (1932), and others   A test of these data on the quantum hit to kill hypothesis will be considered later

Rate of Budding of Irradiated Cells

To determine the effect of the absorbed energy on the rate of reproduction of *S cerevisiae*, counts of the number of cells per colony in both normal and irradiated areas were made at various intervals during the 50 hour period following exposure of the plates   Average values for approximately 100 colonies per area were plotted semilogarithmically against the elapsed time (Fig 3) for a series of exposures at 2652 Å u   In contrast to the typical growth curve shown by the normal cells, cells irradiated with small amounts of energy show a much lower rate of budding with a longer initial lag period and the curve does not flatten out as early as with normal cells   Colonies formed by the irradiated cells continue to lag behind normal colonies and over a 150 hour period appear to reach the resting stage soon after the normal colonies, even though their distribution is such as to permit plenty of nutritive material to be available   Drying out of the medium was prevented by a layer of mineral oil which was found not to inhibit budding of non irradiated cells

Due to the possible presence of inhibitory substances liberated by adjacent dead cells, a complex condition not open to a definite analysis on the basis of the incident energy to which the parent cell was exposed is the more probable state

Oxygen Consumption of Yeast after Irradiation[5]

The contradictory statements in the literature in regard to a stimulative (de Fazi, 1915, 1921), or a destructive effect of ultra violet light (Feuer and Tanner, 1921, Tanner and Ryder, 1923) as measured by the respiration and fermentation of yeast, indicate a need for further

quantitative observations on the gas exchange of these organisms Suranyi and Vermes (1929), also, found an increase of "about 50 per cent" in the oxygen consumption of yeast at pH 7 2

To test this point further, but using monochromatic light of known intensities, a series of observations was made on the oxygen consumption of yeast suspensions immediately after irradiation

Yeast cells from a 24 hour culture incubated as before on malt agar (pH −4 7) at 25°C were washed with about 15 cc of distilled water into a glass cell (3 5 × 2 2 × 2 cm), the exposed side of which was a quartz plate   From this cell 2 cc of the well stirred suspension was pipetted into each of three conical Warburg vessels containing 0 1 cc of 10 per cent glucose solution with 0 1 cc of 10 per cent KOH in the inset   The remainder of the solution was immediately exposed for 5, 20, or 60 minutes to the selected wave-length of ultra-violet light of measured intensity (cf Gates, 1929–30), while stirred constantly by a stream of air   2 cc of this irradiated suspension was pipetted into each of three Warburg vessels containing the same solutions as the controls   All the vessels were then joined on to the manometers of Warburg respirometers (Warburg, 1926) and mounted in the thermostat together with a thermobarometer   The volume of the vessels was about 13 cc , the thermostat was kept at 25°C , and the rate of shaking was maintained at 40 oscillations per minute with a throw of 8 cm   Observations were taken during the $2\frac{1}{2}$ hour period immediately after irradiation   Usually, before starting the respiration tests, portions of the suspension were withdrawn from the irradiated and control specimens, washed in distilled water, and plated on nutrient agar for morphological observations to be made 24 hours later

The significant results of these tests are given in Table III in the order of increasing wave-lengths used   In many of the observations at the different wave-lengths tested such small energies were involved that no effect, either functional or morphological, was observed, these have been omitted from the table   However, in the experiments listed in Table III microscopic examination of the irradiated samples plated on agar and incubated for 24 hours at 25°C showed that from 19 to 87 per cent of the cells had been definitely affected by exposure to single wave-lengths, and up to 96 per cent using the major part of the spectrum   Nevertheless, it will be noted that no observable effects on O_2 consumption were obtained during the $2\frac{1}{2}$ hour period until more than 80 per cent of the organisms had been so affected that normal colonies did not result from their subsequent growth   In only two instances, (3) and (6), did the O_2 consumption drop off to an appreciable extent   In the case of (3) the exposure was the maximum

energy used in the especially effective wave length 2536 Å u   In (6) 96 per cent of the cells showed abnormalities after exposure to the whole spectrum between 2482 and 3132 Å u

In obtaining the percentages of abnormal cells due to irradiation the following types were considered (1) cells producing after 24 hours colonies of less than 16 cells, (2) cells growing to giant size and producing giant daughter cells, often of semi filamentous form, (3) small colonies produced by these giant cells which after the fourth or fifth generation usually produce normal sized cells, (4) single cells considered

TABLE III

No	Wave length	Time exposure	Total incident energy	Abnormal cells	Average O₂ consumption and average deviation		Difference between average values	
					Irradiated	Control	c mm	per ce t
	λ u	*m n.*	*ergs*	*per cent*	*c mm /hr*	*c.mm /hr*		
1	2482	60	28 8 × 10⁴	79	22 0±1 5	21 0±0 3	+1 0	5 4
2	2536	20	27 2 × 10⁴	19	49 0±0 7	49 3±0 8	−0 3	0 8
3	2536	60	81 6 × 10⁴	[87]*	13 2±1 0	17 2±1 6	−4 0	22 0
4	2675	20	21 4 × 10⁴	60	22 7±0 2	21 5±0 7	+1 2	5 35
5	2675	60	64 3 × 10⁴	70	21 5±0 3	20 7±0 5	+0 8	4 10
6	2482 to 3132	60	408 × 10⁴	96	20 0±0 5	25 9±3 1	−5 9	22 80

* Determined by a parallel experiment

to be dead   Cells which later produce two or more buds must be considered alive at the time of the O₂ consumption measurements

Results similar to those indicated in Table III have been obtained by Wels and Osann (1925) for the effect of X rays upon the growth and metabolism of yeast   In certain cases in the present tests the suspensions were left in the respirometers and their O₂ consumption determined 20 hours after the end of irradiation   The same relation between control and irradiated specimens was found as that existing during the 2½ hours immediately following irradiation

It appears from these experiments that a mass of yeast cells so affected by exposure to ultra-violet light that they subsequently reveal functional and morphological abnormalities evidenced by failure to multiply and by giant cell formation, may nevertheless maintain a

normal respiratory rate during the hours immediately following exposure

DISCUSSION

It is evident that whether one or several reactions are involved in the cell changes resulting from irradiation with monochromatic ultra-violet light, yeast cells can undergo different degrees of damage, from which they may or may not recover (1) Inhibition of normal sized colony formation occurs at a low energy level (2) The reproductive mechanism is so damaged that only a few budded cells are produced Growth of the cell, however, continues to the formation of giant cells, which are often associated with an apparent division of the nuclear body (3) At a still higher incident energy the metabolic functions of the cell are injuriously affected as shown by a lowered rate of oxygen consumption (4) Finally, with relatively long exposures, growth and reproduction are stopped The cell may give rise to one bud, increase in size, and apparently "live" for a period of time but ultimately "dies" as indicated by loss of selective permeability to certain dyes, and by cytolysis (deferred death), or it may show no change excepting a more granular appearance of the cytoplasm and degenerative changes (immediate death) (5) The shape of the curves for the lethal action of ultra-violet light on *S cerevisiae* suggests that more factors than (*a*) single quantum hits on several molecules, as suggested by Rahn (1929–30), or (*b*) multiple quantum hits on a sensitive region, as suggested by Wyckoff and Luyet (1931), influence the reaction The age and reproductive stage of the cell at the time of exposure are probable additional factors which modify the course of the process

The results of the respiration tests establish another criterion of the effect of ultra-violet light on microorganisms based on the change of a metabolic process, as compared with criteria based on morphological changes This effect falls between simple inhibition of reproduction and complete killing of the cell as judged by loss of selective permeability of the yeast cell wall (Rahn and Barnes, 1932–33) [6] More

[6] Rahn and Barnes (1932–33) observed that a decrease in the fermentation of glucose by yeast also falls between loss of reproductive power and "death" as measured by loss of selective permeability to Congo Red

observations on the O_2 consumption are necessary before drawing definite conclusions concerning the region of the spectrum in which the effects are greatest, but the data of Table III suggest the possibility that the maximum effect occurs at a different wave length than is found for the morphological effects

I wish to express my gratitude to Professor W J Crozier and Dr Frederick L Gates whose aid and advice made this investigation possible

SUMMARY

Effects of measured ultra-violet light on the yeast *S cerevisiae* have been studied   Methods of culturing and irradiating the yeast, of estimating the nature and extent of the changes produced, and the means used in producing and measuring the radiation are given

No evidence of a stimulative action was observed   The absorbed energy did not produce an all or none effect, arbitrary criteria must be used for judging the various inhibitory and lethal effects   With increase in the incident energy diverse effects were produced until abnormal cell growth and "death" of the cells resulted   Changes in the rate of oxygen consumption did not occur until after a high proportion of the irradiated cells were so damaged that they produced abnormal cells

The shape of the curves relating effect to energy are similar for various wave lengths but different energies are required to produce the same effect at each of the wave lengths studied   The similarity of the curves to that for a first order reaction is noted, but attention is called to the modifying influence of accessory factors such as the age of the cells   A comparison is made of the morphological and metabolic changes on the basis of energy requirements, and their relative value as criteria in judging the effects of ultra-violet light on yeast and physiologically similar microorganisms

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ELECTRICAL RESPONSES FROM THE LATERAL LINE NERVES OF FISHES

V Responses in the Central Nervous System[*]

By HUDSON HOAGLAND

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Descriptions of nerve impulses discharged from lateral line nerves of trout and catfish have been presented in previous papers (Hoagland, 1932–33 a, b, 1933–34 a, b)   A vigorous repetitive discharge of impulses, independent of all external stimulation, has been found to occur over the lateral line nerves—apparently produced by continuous chemical reactions in the sensory cells (neuromasts) of the lateral-line system   The frequency of the impulses has been found to be modified by mechanical and thermal stimulation of the neuromasts

Experiments with certain fresh water fishes other than trout and catfish previously failed to show any nerve impulses in lateral line nerves either of the "spontaneous' variety or in response to external stimulation of the neuromasts   The procedure previously employed involved dissecting free 1 to 2 cm of the nerve and drawing it across electrodes connected to the recording system   It was pointed out (Hoagland 1932–33 b) that this treatment might render very delicate nerves inactive   Heavily scaled fresh water fish that have been examined seem to have tenuous and poorly myelinated lateral line nerves compared to the larger and stronger lateral line nerves of trout which have very small scales, and to those of catfish which have no scales at all.   That this explanation of the absence of activity is probably correct is indicated by the fact that by a modification of the original procedure I have found it possible to obtain the repetitive discharge of impulses, in the absence of external stimulation, in goldfish and in perch   Responses have been obtained from the lateral line nerves of these fish by careful exposure of the nerves and by slipping concentric micro electrodes under them *in situ*   Out of seven goldfish lateral line nerves, three clearly gave the spontaneous responses   Similar responses were obtained from four out of six lateral line nerves of perch

despite the fact that these nerves are extremely small and delicate   Stroking the flank and flexing the the the trunk augmented the responses

By the use of concentric micro electrodes it has been possible to follow the discharge of the lateral-line nerve into the central nervous system of trout, catfish, goldfish, and perch   Twenty-three fishes were tested   The electrodes are made according to the general method first used by Adrian and Bronk (1929)   A fine glass-sheathed silver wire is inserted into the tube of a No 24 hypodermic needle, the silver core thus acting as one lead and the steel needle as the other   The electrodes are connected through resistance-coupled amplifiers to an iron armature oscillograph and to a loud speaker   The silver core electrode was connected to the grid of the first valve and the tube of the needle was grounded

The fishes were either anesthetized with chlorotone or, for the most part, immobilized by severing the spinal cord just posterior to the medulla   Results were the same in both cases   Care was taken to assure against the recording of muscle action currents

A certain small amount of electrical activity was found in some of the fishes in the forebrain and tectum   This may have been due to injury, or to spontaneous central discharges   It did not seem to be correlated with illumination of the eyes   Massive continuous discharges of impulses were found in the tuberculum acusticum, in which the lateral-line nerve terminates   This discharge was silenced on one side by severing the ipsilateral lateral-line nerve but remained on the other side as long as the lateral-line nerve on that side was active   A vigorous discharge of impulses was also found in the cerebellum corresponding to the incoming discharge of lateral-line impulses   The cerebellar activity was diffuse   Severing one lateral-line nerve produced a fairly uniform decline in the impulses throughout all regions of the cerebellum   Cutting both lateral-line nerves silenced the cerebellum as well as the tuberculum acusticum

The spontaneous discharge of trout lateral-line nerves has been found in certain cases to be synchronous (Hoagland, 1933–34 b) The synchronization produces a distinct note from the loud speaker The synchronized discharge was found to enter the tuberculum acusticum where the synchronization ceased, the impulses on reaching the cerebellum being quite arhythmic

When the electrodes were placed in the brain at the place of entrance of fibers supplying the neuromast groups of the head, no evidence of spontaneous discharge was found—suggesting that the

facial neuromasts may have functions different from those along the trunk.

These experiments support the notion that discharges from the lateral line receptors have a tonic reinforcing rôle in postural and swimming reflexes (Hoagland, 1933–34a), in addition to serving as pressure and probably as thermal receptors. The continuous en gagement of groups of neurones in the tuberculum acusticum with the reception of the spontaneous discharge is also suggestive in connection with the demonstrated inhibition of auditory responses of catfish which possess functionally intact lateral line systems (Parker and Van Heusen, 1917)

SUMMARY

Records of spontaneous discharge of nerve impulses, similar to that previously described in catfish and in trout, have been obtained from lateral line nerves of goldfish and perch, by the use of concentric micro electrodes slipped under the nerve *in situ* These impulses have been followed into the central nervous system. They enter the tuberculum acusticum and thence apparently spread diffusely through the cerebellum. Cutting the lateral line nerve on one side silences the ipsilateral tuberculum acusticum, but only reduces the intensity of ipsilateral cerebellar activity. Cutting the remaining lateral line nerve silences activity throughout the tuberculum acusticum and the cerebellum

The maintenance of tonic activity in the tuberculum acusticum by way of lateral line discharge may account for the inhibitory effects of the lateral line system on auditory responses

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THE "BOUND" WATER OF BIOLOGICAL COLLOIDS A REPLY

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The interpretation of the paper by Greenberg and Greenberg (1) has recently been questioned by Bull (2)   In the paper under question it was pointed out, on the basis of the assumption that "bound" water has lost its solvent properties, that the amount of water in this condition in colloidal solutions can be estimated by ultrafiltration experiments with the use of appropriate reference substances   From a series of such experiments it was decided that the amount of bound water associated with such substances as gelatin, casein, and the serum proteins must be quite small

Bull points out that an adsorption of the solute along with the water molecules would invalidate the interpretation offered   After questioning the accuracy of the data, he goes on "Assuming, however, for the moment that the experimental results describe completely the actual conditions, we make the further assumption that 5 per cent of the solute is adsorbed, i e, bound by the substrate—surely a modest estimate "   "   It can be seen that this small correction changes the final values in some cases by over 300 per cent   "   By means of this assumption, Bull shows the possibility of considerable amounts of bound water being present, but not detected   In the original paper this possibility was considered   As was there pointed out, one of the conditions which may invalidate the method is the selection of a reference material which to some extent reacts with or is adsorbed by the colloid   To obviate this as an obscuring factor, a considerable number of reference substances were used in the original experiments   It seems quite a strain upon the assumption advanced by Bull that the reference substances, urea, glucose, KCl, NaCl, and Na_2SO_4, should all be adsorbed by gelatin to an extent just sufficient

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to mask the presence of bound water   The adsorption of the solutes, assumed by Bull, however seemingly modest, would be more convincing if bolstered by an experimental demonstration rather than a mere opinion

No such evidence being offered, a number of experiments were carried out by us designed to detect an adsorption of one of the refer-

TABLE I

Test for Bound Water, and the Adsorption of Glucose in Gelatin and Casein Solutions by Means of Varying Quantities of Glucose as Reference Substance

No	Glucose in original protein free solution per 100 ml	Glucose in ultrafiltrate per 100 ml	Bound water per gm of protein
5 0 per cent gelatin in water + glucose			
	mg	*mg*	*gm*
1	100	101	0 20
2	200	199	—0 10
3	300	301	0 07
4	400	400	0 00
5	500	502	0 08
Average			0 05
6 per cent casein in aqueous solution containing 5 millimols NaHCO₃ and 5 millimols NaCl per 100 ml			
1	100	100	0 00
2	200	200	0 00
3	300	301	0 05
4	400	397	—0 12
5	500	503	0 10
Average less than			0 01

ence materials used, namely glucose, by the proteins, gelatin and casein   An examination of adsorption isotherms and their mathematical equations, makes it highly improbable that a constant fraction of the glucose would continue to be adsorbed as its concentration in the solution is varied   Determinations of the glucose ultrafiltered from a series of protein solutions with varying amounts of glucose then should demonstrate if it is adsorbed even though at some one point

the bound water exactly would counterbalance the glucose adsorbed Such a series with the proteins, gelatin and casein, is given in Table I

EXPERIMENTAL

To avoid uncertain corrections as to non-solvent volume of the proteins the aqueous glucose solutions were first accurately prepared from pure recrystallized glucose and to measured volumes, weighed amounts of purified protein bone dried in an electric oven at 100°, were added   The gelatin was added to mixtures containing only water besides the glucose, but to bring the casein into solution, the aqueous media also contained 5 millimols of NaHCO₃ and 5 millimols of NaCl per 100 ml   The gelatin was dissolved by warming to 50°C and the casein by vigorous agitation   Portions of these protein solutions were now ultrafiltered in the manner previously described (1)   Samples of the ultrafiltrate and of the original aqueous solution were analyzed for glucose by the Hagedorn Jensen method (3)   The analyses were carried out in duplicate, often in triplicate   The estimated accuracy of the analysis is to better than 0 5 per cent

DISCUSSION

The data tabulated in Table I show there was a complete recovery of the 100 mg of glucose per 100 ml of solution added to each increasing step in the series   Over a fivefold increase in the glucose concentration, there is no indication whatsoever of an adsorption of glucose by the proteins   The net amount of bound water per gram of each protein, calculated in the last column of the table, while positive in value, is very small in amount   From this test, the assumption of Bull does not appear tenable, and if any amount of glucose is adsorbed by either of the two proteins, it must be small indeed

The probability of an adsorption or association of some water with the proteins and other lyophilic colloids has not been denied by us However, our evidence indicates it to be far below the amount claimed A rough picture of the probable order of magnitude of the bound water can be gained from an examination of the molecular dimensions of the proteins   From modern work on surface adsorption, initiated by Langmuir (4), no more than one or two monolayers of water molecules would be expected to be adsorbed by the proteins with such force as to lose their solvent properties   Furthermore, the whole of the protein surface would hardly be expected to be the seat of such an adsorption, but rather only the polar portions   If the protein hemoglobin is used

as a model (molecular weight 66,700, density 1 3), from Avogadro's number, there is calculated, assuming the protein to be spherical, the molecular diameter 54 5 Å u and the volume 85,000 Å u ^{3} By a similar calculation, water molecules have a diameter of 3 85 Å u and a volume of 30 Å u ^{3} If the water molecules are considered to be spheres packed over the spherical surface of the protein, then about 300 water molecules are required to give a complete surface layer of water 1 molecule thick   Assuming the water molecule to be a cube, about double this number can be so packed   The first consideration would lead to a value of 0 08 gm of bound water per gram of protein for each monolayer of water, the latter about 0 16 gm   However, since, as has already been mentioned, the total protein surface can hardly be adsorptively active for water, these amounts must be reduced to some fractional values, a good deal less   Such a consideration leads to the view then that an adsorbed water layer a number of molecules thick on the proteins would hardly yield a detectable amount of bound water

SUMMARY

The objection by Bull to the estimation of bound water by ultra-filtration, because of an assumed adsorption of the reference substance, has been found invalid for glucose   No adsorption of glucose by the proteins, casein and gelatin, could be detected

The estimation of the bound water of proteins from the probable surface adsorption of water by proteins leads to only a small value

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ON THE THEORETICAL SIGNIFICANCE OF TALBOT'S LAW

By WILLIAM ARNOLD AND C P WINSOR

(From the Laboratory of General Physiology, Harvard University Cambridge)

Stimulation of a photoreceptor by intermittent light produces a sensation of continuous brightness if the frequency of interruption is sufficient   Talbot's law states that the effect of such intermittent light of intensity I is identical with that of continuous light (having the same spectral composition) of intensity I_c if

$$\alpha I = I \qquad (1)$$

where α is the fraction of a complete cycle during which the intermittent light acts   Experiment indicates that Talbot's law holds within the limits of experimental error over a wide range of intensities It is the purpose of this note to show exactly what this means for a theory of photoreception

The assumptions which we shall make about the nature of the photo receptive process are essentially similar to those which Hecht has made in numerous papers [1]   We have chosen, however, to express them in as general a form as possible, in order to provide the broadest possible theoretical basis for deduction

We assume, first, that the state of the photoreceptor at any instant is completely determined by its previous history in respect of exposure to light   This assumption needs no special justification, clearly, it is implicitly made by all experimenters

Second, we assume that photoreception involves two processes, essentially opposite in direction, one of which is sensitive to light, the other independent of light

As we have phrased it, this assumption makes no specific statement about the physics or chemistry of the photoreceptor   The assumption itself is rendered plausible by the facts of light- and dark adapta-

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[1] Hecht S   J Gen Physiol , 1918–33

tion and the steady state Its eventual justification must rest on its ability to explain and predict the results of experiment We should like to emphasize the fact that we do not imply that a complete account of photoreception can be given in terms of only two processes We are convinced of the contrary, but we feel that the simplest and most general mechanism should be postulated initially

A third assumption is necessary to describe the relation of the photoreceptor mechanism to sensation It seems clear that sensation must in some way be connected with the rate of the photosensitive process The facts of persistence of images show, however, that sensation is not determined by the value of this rate at any single instant We must rather assume that the rate of the photosensitive process at any instant affects sensation over a period of time, and that therefore sensation is somehow determined by the value of the rate over a previous period of time For our present purposes it is not necessary to make our assumption more precise, since we shall be dealing entirely with conditions under which the sensation produced is essentially constant, we need only assume that sensation is determined by the integral of the rate of the photosensitive process taken over a single cycle

We may now express these assumptions mathematically
Let

I = light intensity
$x, y, z,$ = numerical values of the variables other than light intensity which determine the rates of the processes in the photoreceptor,
$v_1 = \phi_1(I, x, y\ )$ = rate of the photosensitive process,
$v_2 = \phi_2(x, y\ )$ = rate of the "dark" process

From our first assumption it follows that there exist functional relations between the variables x, y, z,   , which will allow us to eliminate all but one of these from the expressions for the rates, so that we may write without loss of generality

$$v_1 = \phi_1(I,\ X) \text{ and } v_2 = \phi_2(X)$$

While light falls on the photoreceptor, the net rate of change of X will be

$$\frac{dX}{dt} = \phi_1(I,X) - \phi_2(X), \tag{2}$$

and when no light falls on the photoreceptor, the rate of change of X will be

$$\frac{dX}{dt} = -\phi_2(X) \tag{3}$$

Let us now suppose that the eye is exposed to intermittent light, the duration of a light flash being $\alpha\Delta t$, and the interval between flashes $(1-\alpha)\Delta t$ Then (if Δt is short enough so that we may neglect second derivatives) we have during a light flash

$$\Delta X_L = \alpha\Delta t(\phi_1(I,X) - \phi_2(X)), \tag{4}$$

and during a dark period

$$\Delta X_D = -(1-\alpha)\Delta t\,\phi_2(X) \tag{5}$$

The net change in X over a complete cycle is $\Delta X_L + \Delta X_D$, which is zero when the steady state is reached, so that we have, after obvious simplifications

$$\alpha\phi_1(I, X) - \phi_2(X) = 0 \tag{6}$$

In continuous light, when the steady state is reached, we have

$$\phi_1(I,\lambda\,) - \phi_2(\lambda\,) = 0 \tag{7}$$

Let us now suppose that the conditions of the experiment are such that the sensations produced by the two lights are equal   By our third assumption we may write

$$\text{Sensation} = f\left(\int_0^{\Delta t} \phi_1(I,X)dt\right) \tag{8}$$

If the period Δt is sufficiently short, we may write this as

$$S = f(\phi_1(I\,X)\Delta t) \tag{8'}$$

The function f we assume single valued and monotonic, at least within the range considered   (Were this not the case, we should have the possibility that an increase in illumination would result in a decrease in the sensation of brightness)

In the case of flashing light, the sensation over a period is

$$S_f = f(\phi_1(I\,X)\alpha\Delta t), \tag{9}$$

and in continuous light

$$S_c = f(\phi_1(I_c, X_c)\Delta t) \tag{10}$$

If sensation in the two cases is equal, we have clearly

$$\phi_1(I, X)\alpha \Delta t = \phi_1(I_c, X_c)\Delta t \tag{11}$$

We now observe that (11), together with (6) and (7), allows us to write

$$\phi_2(X) = \phi_2(X_c), \tag{12}$$

and if, as it is reasonable to assume, ϕ_2 is a single valued and monotonic function, we have

$$X = X_c \tag{13}$$

Talbot's law states that (11) holds only if $I_c = \alpha I$ Using this and (13) we have

$$\alpha\phi_1(I, X) = \phi_1(\alpha I, X) \tag{14}$$

Now (14) is a functional equation of the form

$$f(xy) = xf(y)$$

Setting $y = 1$ we have as the solution of this equation

$$f(x) = xf(1) = Cx$$

It therefore follows that the rate of the light-sensitive process must be of the form

$$r_1 = I\phi_1(X) \tag{15}$$

Expressed in words, equation (15) shows that Talbot's law has as a necessary consequence that the rate of the photosensitive process is directly proportional to the intensity of the incident light This result is completely independent of any assumption we may be led to make as to the manner in which the light and dark processes involve other variables than light

SUMMARY

On the assumptions (1) that the state of the photoreceptor is completely determined by its previous history in respect of exposure

to light, (2) that photoreception involves two opposed processes, one of which is light sensitive, and (3) that sensation is determined by the rate of the light-sensitive process integrated over a short period, it is shown that Talbot's law has as a necessary consequence that the velocity of the light sensitive process must be directly proportional to the intensity of the stimulating light

THE RATE OF ESCAPE OF HEMOGLOBIN FROM THE HEMOLYZED RED CORPUSCLE

By HUGO FRICKE

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In a recent note[1] Ponder has presented some preliminary results of an experimental study of the rate of escape of hemoglobin from hemolyzed red corpuscles These experiments are of interest in furnishing a means of determining to what extent the hemoglobin is free to leave the corpuscle In particular, assuming that the resistance is at the surface of the corpuscle, the permeability of the surface of the hemolyzed corpuscle may be calculated

In the following discussion the assumption is made that the escape of the hemoglobin is due solely to its diffusion, neglecting any influence of gravity or convection currents, which appears wholly justified in view of the small size of the corpuscle and of the experimental conditions under which the escape is measured

We may start by assuming that the surface of the corpuscle becomes completely permeable, and shall calculate the change of concentration of hemoglobin as a function of time, at any particular point, which takes place as a result of the diffusion The corpuscle may be assumed to be spherical, since it appears[2] that a change to this form always takes place before hemolysis occurs We shall furthermore assume that Fick's law holds, with a constant value (D) of the coefficient of diffusion The diffusion of hemoglobin does decrease with increasing concentration,[3] but to too slight an extent to require its consideration for the present purpose

The increase of hemoglobin inside a spherical shell, thickness dr,

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[1] Ponder, E , Proc Soc Exp Biol and Med , 1934, **31**, 562

[2] Ponder, E The mammalian red cell and the properties of haemolytic systems Protoplasma Monographien, Berlin, Gebrüder Borntraeger, 1934 6

[3] Zeile, K , Biochem Z , Berlin 1933 **258**, 347

during the time dt, is equal to the difference between the amount of pigment entering and leaving the shell   The latter quantities are obtained from Fick's law   The following differential equation expresses this equality

$$4\pi r^2 \frac{dc}{dt} = D \frac{d\left[4\pi r^2 \frac{dc}{dr}\right]}{dr}$$

from which

$$\frac{d(rc)}{dt} = D \frac{d^2(rc)}{dr^2}$$

with the boundary conditions as follows

$t = 0, c = c_o$ from $r = 0$ to $r = \rho$, and $c = 0$ from $r = \rho$ to $r = \infty$, where c is the concentration of hemoglobin at a distance r from the center of the corpuscle and at a time t, and c_o is the initial value of c The radius of the corpuscle is ρ The solution is

$$c = \frac{c_o\sqrt{Dt}}{\sqrt{\pi}\, r}\left[e^{-\left(\frac{\rho+r}{2\sqrt{Dt}}\right)^2} - e^{-\left(\frac{\rho-r}{2\sqrt{Dt}}\right)^2}\right] + \frac{c_o}{2}\left[\Phi\left(\frac{\rho+r}{2\sqrt{Dt}}\right) + \Phi\left(\frac{\rho-r}{2\sqrt{Dt}}\right)\right]$$

Φ represents Gauss' function

Introducing $\frac{c}{c_o} = C$, also $\frac{r}{\rho} = R$, and $\left[\frac{2\sqrt{Dt}}{\rho}\right]^2 = T$, (1) we have

$$C = \frac{1}{2\sqrt{\pi}}\frac{\sqrt{T}}{R}\left[e^{-\frac{(1-R)^2}{T}} - e^{-\frac{(1-R)}{T}}\right] + \tfrac{1}{2}\left[\Phi\left(\frac{1+R}{\sqrt{T}}\right) + \Phi\left(\frac{1-R}{\sqrt{T}}\right)\right] \quad (2)$$

The average concentration of hemoglobin, along a radial line, is

$$C_m = \int_o^\infty C\ dR \quad (3)$$

Equations (2) and (3) are solved by numerical calculation   Fig 1 shows C_m as a function of T

Ponder s observations are for the corpuscles of man   Taking the volume of this corpuscle as $89\mu^3$, the value of ρ is $\rho = 2\,8 \times 10^{-4}$ cm Measurements of the coefficient of diffusion of hemoglobin by Sved-

berg and Nichols,[4] by Northrop and Anson,[5] and by Zeile[3] are in substantial agreement, giving as an average, over the range of concentrations of hemoglobin from 0 to 30 per cent $D = 7 \times 10^{-7}$ cm²/sec (20°C). For a value of $C = 0.10$, we obtain from Fig 1 $T = 6$. Therefore, from (1) $t = 0.16$ sec

The much longer times of 2 to 6 seconds, found by Ponder[1] for hemolysis with dilute saponin, indicates a considerable impermeability of the surface of the hemolyzing corpuscle
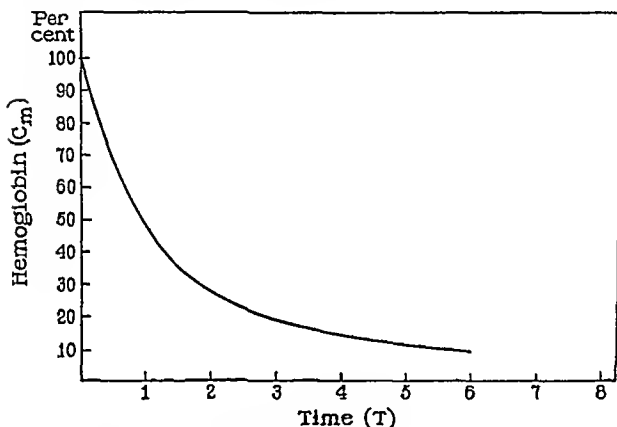
FIG 1 The escape of hemoglobin, as a function of time, from a completely permeable erythrocyte

For stronger saponin, the method used by Ponder cannot be used, because it requires a fairly long latent period. That the escape becomes more rapid as the concentration of the lysin is increased, may be concluded by observing the speed with which a suspension of cells hemolyzes when strong saponin is added. It is quite possible that a time of escape as short as the theoretical value may be at

[4] Svedberg T, and Nichols, J B, *J Am Chem Soc*, 1927, 49, 2920
[5] Northrop J H and Anson, M L *J Gen Physiol* 1929 12, 543

tained, indicating complete permeability of the surface of the hemolyzing corpuscle   Measurements of the electric conductance[6] lead to a similar view   Cells hemolyzed with mild lysins (water, complement-amboceptor, saponin in low concentration) were found to have a conductance too low to measure, while with strong saponin a complete permeability of the cells to the electric current is produced

When the time of escape is as long as that found by Ponder, the concentration gradient of the pigment is practically all at the membrane of the corpuscle   The permeability μ_H of the membrane to hemoglobin is defined as the amount of pigment passing through unit area of membrane, in unit time, per unit difference of concentration   The decrease of hemoglobin inside the corpuscle is equal to the amount which has diffused through the membrane   The latter quantity is obtained from Fick's law   The following equation expresses this equality

$$\frac{4}{3}\,\pi\rho^3\,dc = \mu_H \times c \times 4\pi\rho^2\,dt$$

from which

$$c = c_o\,e^{-\frac{3\mu_H t}{\rho}}$$

$$\mu_H = \frac{\rho}{3t}\,\log_e\frac{c_o}{c}\ \text{cm /sec.} \tag{4}$$

Using Ponder's average time of 4 seconds for $\frac{c}{c_o} = 0\ 10$ with $\rho = 2\ 8 \times 10^{-4}$ cm , we obtain $\mu_H = 5 \times 10^{-5}$ cm /sec   This value of μ_H is for a concentration of saponin which produces complete lysis of human cells (in 1 per cent NaCl) in about 3 minutes

Claim has been made[7] that the hemoglobin leaves through one or more holes in the membrane   It may be of interest to calculate, for any particular number of holes, how large each hole must be to give a

⁶ Fricke, H , and Curtis, H J , The electric impedance of suspensions of biological cells, in Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, Long Island Biological Association, 1933, 1, 117
⁷ Ponder, p 85

certain rate of escape   With N holes (diameter d) placed so far from each other that there is no appreciable interference, the value of μ_H is

$$\mu_H = \frac{0\,96 \times d \times D \times N}{4\pi\rho^2} \tag{5}$$

This expression may be obtained from a formula giving the solution of a parallel problem in electric conductance [8]   Using $\mu_H = 5 \times 10^{-5}$ cm /sec , $D = 7 \times 10^{-7}$ cm²/sec , and $\rho = 2\,8 \times 10^{-4}$ cm , we obtain

$$d = \frac{0\,7}{N} 10^{-4} \text{ cm}$$

CONCLUSIONS

A theoretical treatment is given of the rate of escape of hemoglobin from the hemolyzed red corpuscle   For complete permeability of the surface, as may perhaps be produced by strong lysins, the time taken for the hemoglobin to decrease to 10 per cent of its original concentration is calculated to be 0 16 seconds (for the human cell)   For dilute saponin, giving complete lysis of human cells in 3 minutes, Ponder found a time of escape of 4 seconds, from which the permeability of the membrane to the pigment is calculated to be $\mu_H = 5 \times 10^{-5}$ cm /sec

[8] Jeans J H , Electricity and magnetism Cambridge University Press, 1915, 356

THE CHLOROPHYLL UNIT IN PHOTOSYNTHESIS

By WILLIAM ARNOLD and HENRY I KOHN

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Emerson and Arnold (1) have shown that for each molecule of carbon dioxide reduced per flash of light by the green alga *Chlorella pyrenoidosa*, approximately 2,500 molecules of chlorophyll are present [1] This suggests the existence of a chlorophyll unit in the photosynthetic mechanism Before such a suggestion may be accepted, however, at least four possible criticisms must be met

I There may be a large error in the determination of the oxygen production by means of the Warburg Barcroft apparatus

II There may be a large error in the determination of the chlorophyll content by means of the spectrophotometer

III *Chlorella pyrenoidosa* may be unique in having such a large ratio

IV The theory upon which rests the analysis of photosynthesis by means of flashing light (2) may be inadequate

The purpose of this paper is to discover whether any of the foregoing forbids the assumption of a chlorophyll unit

I

To check the accuracy of the manometric determinations, oxygen was produced by electrolysis in a specially adapted Warburg vessel at a rate approximately equal to that obtained in experiments on photosynthesis It was found that the rate determined manometrically agreed to within 10 per cent with that calculated by Faraday's laws These experiments were performed with Mr C S French

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[1] The conditions of their experiments were such that the cells, suspended in carbonate buffer, were illuminated intermittently by short flashes (less than 1×10^{-4} sec), separated by dark periods adequate for the restitution of the photochemical reaction to maximum sensitivity Light saturation and carbon dioxide saturation were maintained

II

The spectrophotometric estimation of chlorophyll was checked by determining the value of the extinction coefficient of a standard solution, containing 10 mg of chlorophyll per liter of methanol, according to the method previously described (1)   Two monochromatic sources were used, neon—6598 Å , and helium—6678 15 Å , of which the latter can be made the brighter   The chlorophyll preparations, from horseweed and spinach, had been extracted in Professor O L Inman's laboratory at Antioch College, and were obtained through the courtesy of Dr Emma M Dietz   In Table I the values of the extinction coefficient obtained are compared with the value previously published by Emerson and Arnold (1)   No significant differences are apparent

TABLE I

Extinction Coefficients for Methyl Alcohol Solutions Containing 10 Mg of Chlorophyll per Liter

Chlorophyll solutions	Neon $-6598\ 95$ Å	Helium $-6678\ 15$ Å
Chlorella (Emerson and Arnold (1))	0 476	—
Spinach	0 485	0 449
Horseweed	0 486	0 459

For the preparations of chlorophyll used above, the value of the ratio of chlorophyll *a* to chlorophyll *b* is about 3 to 1   Since approximately this value holds for most plant material, the extinction coefficients determined may be generally applied   The chlorophyll estimations thus made will not be absolute, but such errors as are resident in them cannot decrease markedly the computed size of the chlorophyll unit

III

Since the high value of the ratio, mols of chlorophyll/mols of carbon dioxide reduced per flash, may be a unique property of *C pyrenoidosa*, we have determined its value for six species of plants drawn from four phyla, by experimental methods similar to those utilized by Emerson and Arnold (1)   The results, summarized in Table II, indicate

that the value of the ratio, designated ρ, normally falls between 2,000 and 4,000   Adverse culture conditions and ultraviolet radiation can

TABLE II

Values of the Ratio ρ, Mols of Chlorophyll/Mols of Carbon Dioxide Reduced per Flash, for Six Species of Plants

Plant	Position of material in respirometer vessel	Temperature	Ratio
		C	
Bryophyllum calycinum			
1	Leaf floated on surface of carbonate buffer*	31 5	2 500
2	"	31 5	2 600
Chlorella vulgaris	Suspended in buffer	25 0	2 800
Lemna sp			
1	Floated in buffer	25 0	2 600
2	"	25 9	3 200
3	"	29 1	2,900
Nicotina langsdorffii (flowers)			
1	Supported upright in well partly filled with tap water surrounding air space partly filled with buffer	29 9	2 800
2		29 2	2,500
Selaginella sp	Stem in tap water in side arm leaves projected above buffer in air space	28 3	4 200
Stichococcus bacillaris			
1	Suspended in buffer	25 0	3,700
2	"	29 7	5 000

* Carbonate buffer composed of 15 parts 0 1 м $K\ CO_3$ plus 85 parts 0 1 м $KHCO_3$

increase the value of ρ, but in no case have we been able to decrease the value to a small number

IV

The significance of the high value of p, that a large number of chlorophyll molecules are present for each molecule of carbon dioxide reduced, is dependent upon the validity of the assumptions made in the analysis of photosynthesis under flashing light (2)   The most important of these, for our present purposes, is that which dictates the availability of all chlorophyll for photochemical work at the beginning of each flash   However, if the life-time of the chlorophyll reaction should extend over the period occupied by a sufficient number of flashes and their accompanying dark-times, the value of p would become a small number, e g , 10   But this possibility is rendered unlikely by the experiments of Warburg (3), who found the light efficiency of photosynthesis in *Chlorella* to be well over 50 per cent   Since the efficiency is high, a life-time long enough to reduce the value of p would imply so large a change in the absorption coefficient of chlorophyll during photosynthesis that it could not have escaped detection

SUMMARY

In six species of plants, representing four phyla, the minimum number of chlorophyll molecules present for each molecule of carbon dioxide reduced appears to lie between 2,000 and 3,000   This finding suggests the existence of a chlorophyll unit

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TEMPERATURE CHARACTERISTICS FOR METABOLISM OF CHLORELLA

I The Rate of O_2 Utilization of Chlorella pyrenoidosa with Added Dextrose

By W J CROZIER P S TANG and C S FRENCH

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I

The unicellular alga *Chlorella pyrenoidosa* has been extensively studied with respect to its photosynthetic activities (*cf* Warburg, 1928, Emerson, 1928–29, Emerson and Arnold, 1931–32, Arnold, 1933–34) It exhibits a measurable rate of respiration in Knop solution without glucose, this respiration seems to differ from the more active respiration apparent when glucose is added to the medium (Emerson, 1926–27), as the two behave differently toward inhibitors (HCN, H_2S, CO) With this organism, then, it should be possible to obtain temperature characteristics (Crozier, 1924–25) for the velocities of the photosynthetic reactions, for several aspects of respiration, and for growth (multiplication) For these processes, also, the nature of the temperature relations may be investigated through the influence of alterations of the medium upon them In this way desirable in formation may be secured as to the meaning of the temperature char acteristic, μ of the equation

$$\text{velocity} = ke^{-\frac{\mu}{RT}}$$

if the relationship is found to provide a satisfactory description of the measurements (*cf* Crozier, 1924–25, *et seq*, Stier, 1932–33) Data suitably obtained should then give some information as to the prob ability that the reproducible constant μ may have reference to a specific property of a relatively simple chemical system controlling the

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rate of a measured process (*cf* Crozier, 1924–25, Crozier, Stier, and Pincus, 1929, Pincus, 1930–31) We know that exposure to ultra-violet radiation may be used to induce decay of photosynthetic activity in *Chlorella* without simultaneous interference with respiration (Arnold, 1933–34) It may be found that critical temperatures and other features of the curves relating speeds of diverse respiratory activities of *Chlorella* to temperature are notably different Such findings would be effective in support of the general conception that the kinetics of relatively uncomplicated controlling catalyzed processes in living cells may be adequately characterized in specific respects We shall deal first with respiration (O_2 utilization) by *Chlorella* in Knop solution containing 1 0 per cent glucose

II

A pure culture of *Chlorella pyrenoidosa* from the same stock as that used for studies on photosynthesis by Emerson and Arnold (1931–32) was kindly supplied to us It was grown in Knop solution   The flasks (Warburg and Negelein, 1922) were about 300 ml in capacity, glass-sealed except for the openings of the inlet and outlet tubes provided for the passage of a gas mixture   The latter was 5 per cent CO_2 in air, stored in a commercial gas cylinder and allowed to bubble very gently through the cultures   The flasks were placed in a large crystallizing dish 30 cm above three 65 watt frosted lamps, with a stream of water passing constantly through the dish to keep the temperature surrounding the cultures at about $20° \pm 2°$

The culture solution is the modified Knop solution as used by Emerson (1926–27)   Stock solutions were made up of

> (A) $MgSO_4$ $7H_2O$, 50 gm per liter
> (B) KNO_3, 25 gm per liter
> (C) KH_2PO_4, 25 gm per liter
> (D) $Ca(NO_3)_2$, 11 3 gm per liter
> (E) $FeSO_4$ $7H_2O$, 0 336 gm per liter

gravitational force of 800 in a 100 ml. flask for 5 minutes at a time   Under such
conditions, a culture 7 to 10 days old gives about 500 c.mm. of cells [1]

A 2 ml portion of the suspension containing about 20 mm [1] of cells was pipetted
into a conical vessel of the Warburg respirometer   The vessel used was about 15
ml in capacity with a side arm and with an inset for alkali   In our experiments
the inset contained 0 2 ml of a 10 per cent NaOH   The vessels had been soaked
in chromate sulfuric acid mixture overnight, and were repeatedly rinsed with
tap water, and then with distilled water, finally they were filled with distilled water
and allowed to stand for at least 10 minutes   They were dried in an oven main
tained at 105°   A ring of paraffin was smeared around the inner edge of the inset
to prevent the creeping of the alkali into the main portion of the vessel   The
vessels, after being connected to the manometers, were placed in thermostats
the temperature of which may he maintained to within ±0 01° (described by
Stier and Crozier, 1932–33)   The respirometers were shaken at a speed of 70
complete oscillations per minute with a throw of 8 cm   Preliminary experiments
showed that the rate of O consumption was independent of shaking speed between
40 and 100 oscillations per minute   The experiments were performed in darkness
and the readings were made with a weak neon lamp so arranged that the light fell
on a spot on the manometers   At least 30 minutes were allowed for thermal adap
tation at each temperature before the stop-cocks of the manometers were closed
and the first readings taken   Readings were taken thereafter at about $\frac{1}{2}$ hour
intervals for at least 2 hours and sometimes for 10 hours at a given temperature

The temperature was varied in two ways   In certain series of experiments, two
to four thermostats were operated at once, each at a different temperature, and the
rates of O_2 consumption by equal quantities of cells from the same suspension were
compared   In other series of experiments only one thermostat was used and the
cells were subjected to successive changes of temperatures throughout the range
used   For instance, in one series, the rates of O_2 consumption were observed for a
certain length of time at 15°   Without disturbing the experimental set up, the
temperature of the tank was lowered to 10°   After a period of 30 minutes for
thermal adaptation, during which the stop cock of the respirometer was turned
so that the system was open to the air the cock was closed again and readings
were resumed for another period   At the end of this period the temperature was
lowered to 5 0° and the process was repeated   After a period of 5° the tempera
ture was raised to 7 5°, to 12 5 , then to 17 5°, 22 5°, and finally to 25°, and read
ings were made at these temperatures after sufficient time had been allowed for
thermal adaptation

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[1] The apparent volume of cells in a given suspension changes with conditions of
centrifuging   The volumes obtained are given merely for rough comparisons
and should not be taken for computation of absolute rates

III

It is necessary to show that at constant temperature the rate of respiration is sufficiently constant throughout the period of observa-
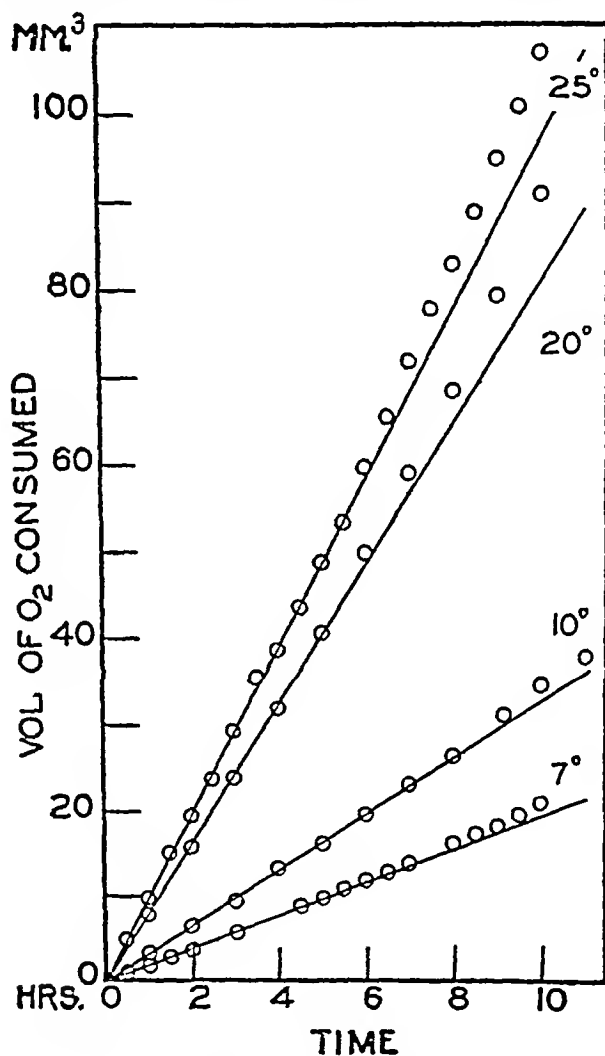

Fic 1 Amounts of O_2 consumed by *Chlorella* cells are plotted against time, four typical experiments The rate of utilization of O_2 is constant for about 5 hours

tion (Crozier and Navez, 1930–31, Tang, 1930–31, Stier, 1932–33) and that it is not appreciably influenced by previous sudden changes in temperature

A number of experiments were performed at various constant temperatures for about 10 hours to establish the constancy of rate for the duration of our experiments Fig 1 gives the results of four representative experiments, at 7 , 10°, 20°, and 25° respectively The rates are constant for about 5 hours, after which they rise, due probably to increase in the number of *Chlorella* cells (or to growth of bacteria) Measurements made for shorter periods than 5 hours are then reliable within the errors of the method

The controversial "stimulating' effect of alternating temperature on life processes has often been reported and discussed Kostychev (1927) gives a review of this matter as it concerns respiration (*cf* Crozier and Navez, 1930–31, Tang, 1930–31) To see whether sudden changes in temperature had any special effect upon O_2 consumption by *Chlorella*, a series of tests was made Three thermostats

TABLE I

Effect of Sudden Changes in Temperature upon Rate of O_2 Consumption by Chlorella pyrenoidosa

Temperature change	n	n	n
18–13	1 0	1 1	1 0
13– 8°	1 0	1 2	1 0
13–18	1 0	0 9	1 0
8–13°	0 9	0 95	1 0

n' is the ratio of the rates of the cultures subjected to changing temperatures (hereafter called $E\ C$) to those of the control cultures ($C\ C$) in the first period when they were at the same temperature, n is the ratio of the rates of $E\ C$ to $C\ C$ when the former were changed to a lower or higher temperature n'' is the ratio of $E\ C$ to $C\ C$ when the former were returned to the original temperature in the third period

were operated at 8°, 13°, and 18° respectively Fourteen samples of *Chlorella* cells all from the same suspension, were distributed as follows four each at 8° and 18°, and 6 at 13° After a period of respiration of about 2 5 hours, two cultures from the 13° group were transferred to 8° and two to 18° In return two cultures each from 8° and 18° were placed at 13 After a second period of respiration, sufficient time being allowed for thermal adaptation in all cases the cultures were again interchanged so that they were at the same temperatures as they had been at the beginning of the experiment. Thus we have a set of two control experiments at each temperature which respired at constant temperature for a long time, and two cultures which were first subjected to a given temperature then to a lower or higher temperature and were finally returned to the original temperature In all cases the rates for the cultures which were returned from a sojourn at a higher or lower temperature for a period of about 2 5 hours were very nearly the same as

those of the controls which remained undisturbed at the initial temperatures
The rate of O_2 consumption of a culture transferred from a higher temperature to
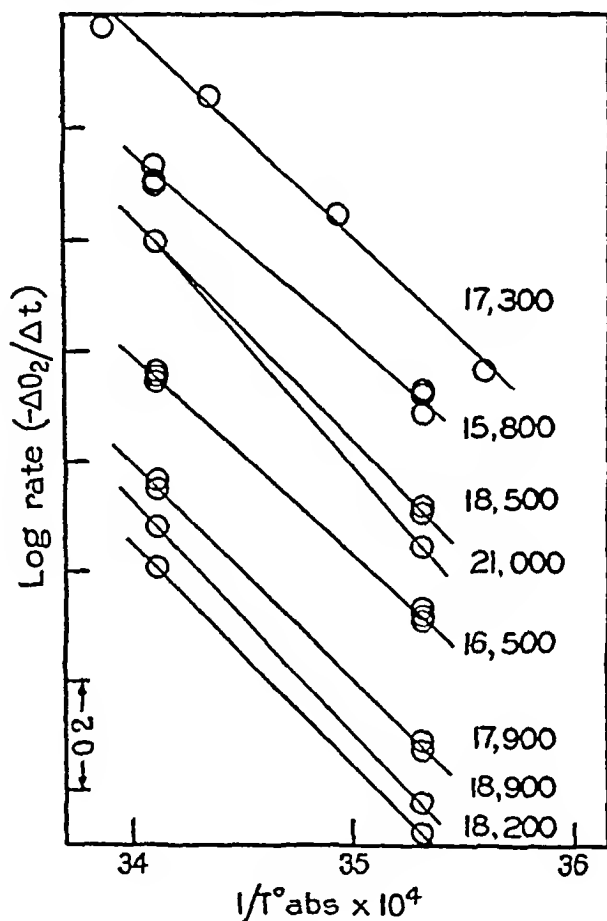
FIG 2

FIGS 2 and 3   Log relative rates of O_2 consumption by *Chlorella pyrenoidosa* is
plotted against $1/T$ abs $\times$ 10^4 Each line represents a series of experiments
The order in which the points were taken is either marked by numbers attached
to the groups of circles or by different symbols   In the latter case the solid circles
represent the experiments made during the first period and the open ones are
those made in the second period (see text)   The values of μ are given along the
individual lines, see text   Fig 2 represents points taken from the data of Tang
and French (1933–34) obtained in connection with measurements made at various
O_2 tensions

to a higher temperature, the rate is slightly lower than that for a culture at the higher temperature from the beginning The differences are small and but for their consistency appear to be within the limit of error The results are summarized in Table I

When proper precautions are taken, that is, when sufficient time has been allowed for thermal adaptation, and when the experiments are not performed over too long a period at a given temperature, the slight variation of the rates with time and with sudden changes in temperature may be considered negligible
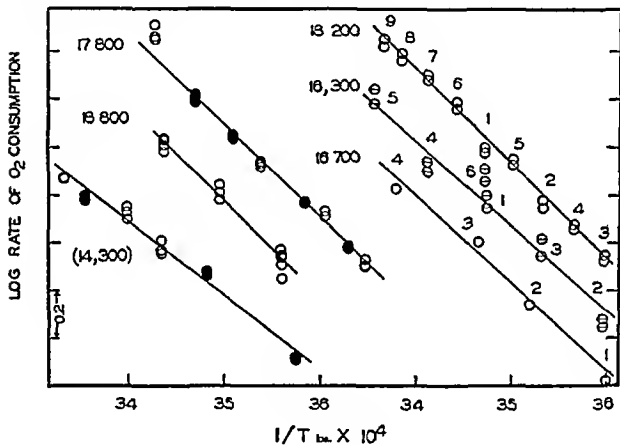
Fig 3

IV

We may now consider the results obtained when *Chlorella* cells respire at temperatures ranging from 1° to 27° Fourteen series of experiments were performed altogether, with the two methods already described All the data are plotted individually in Figs 2 to 4, the ordinates give the logarithms of the relative rates of O_2 consumption, the abscissae give $1/T$ abs $\times 10^4$ Each series consists of 1, 2, or 3 experiments, all the points are plotted in the figures Whenever neces

sary the order in which the points were obtained is given by the numbers attached to the groups of points, or by the way in which the points are represented   In any given series, the solid circles are points obtained in the first period and the open circles represent points which were obtained at the second period   The values of μ, the temperature characteristic (Crozier, 1924–25), are given along the individual lines   It is seen that the values vary from series to series falling between 14,300 and 21,200

Observations with *Chlorella* cultures, under the conditions we have described, are open to a number of sources of confusion   From much earlier work we know that determinations of μ from readings at two or three temperatures are subject to the possibility of a considerable uncertainty arising from the natural latitude of variation found in successive or repetitive determinations at the same temperature (*cf* Crozier and Stier, 1924–25, Stier, 1932–33), this variation may be of different relative magnitudes on either side of a critical temperature (*cf* Crozier and Stier, 1926–27, Stier, 1932–33)   Even were the sometimes disturbing effects of the sensitivity (as contrasted with the accuracy) of the manometric method completely avoided, we cannot be certain that all the cells in a given culture will respond in the same manner to transitions from one temperature to another   For such reasons relatively considerable variation must be expected in the apparent value of μ computed from determinations at only two temperatures (*cf* also Lineweaver, Burk, and Horner, 1931–32)   It is therefore necessary to combine the several series of determinations, to obtain the most probable value of the temperature characteristic

All the data for the fourteen series of experiments were brought together by shifting the lines in Figs 2 and 3 to a common level at 15°C by proper factors, giving the mass plot of Fig 4   The points show a considerable degree of scatter, greater at higher temperatures   A line is drawn through the points indicating the general trend of the change of the rates with temperature   The slope of that line gives the value $\mu = 19,000$ cal   The points are relatively quite scattered, more so at the higher temperatures, this may be due, in part, to special effects at the higher temperatures, or to a real difference in the latitude of variation above a critical temperature at 20°±   The points do not fall within parallel lines as they do in many other instances (*cf*

Crozier *et al* [2]), this indicates that technical errors enter in this case to a relatively large extent

The highest individual value of μ is roughly 21,000, based, however, on two points only (Fig 2), the lowest, 14,300, shown in Fig 3, can-
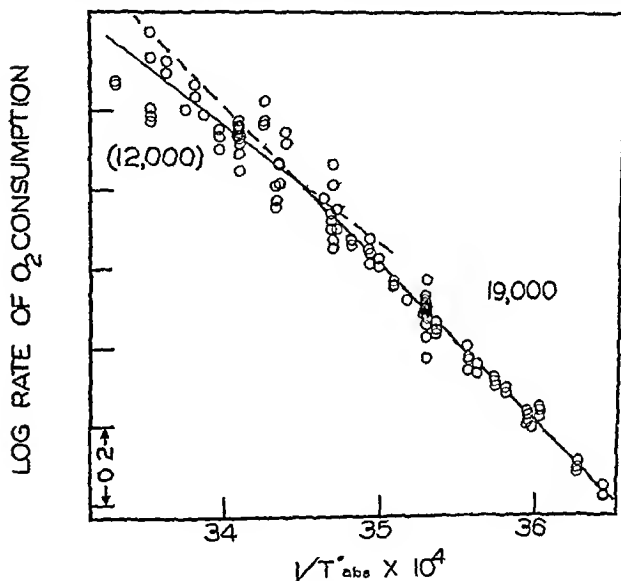
FIG 4 All the series of experiments presented in Figs 2 and 3 are brought together by shifting the lines to a common level at 15°   The coordinates are the same as those used in Figs 2 and 3   The points scatter about a best fitting line giving a μ of 19 000 cal   (The probable change of slope above 15° is discussed in the text.)

not be so easily discarded   All other values lie within the range 15,800 to 19,000, on the basis of the individual series (Fig 3)   The apparent individual slopes (Figs 2, 3) may be disturbed by the oc

* Crozier *et al* , *J Gen Physiol* 1924 to date

currence of a break, more pronounced in some sets than in others, at about 20° Further investigation at higher temperatures would be necessary to clear up this matter definitely The true value of the temperature characteristic may not be far removed from that given by the line fitted in Fig 4 If we arbitrarily weight each series according to the number of vessels and to the spacing of the temperature, multiply each by its rating and divide the sum of the figures thus obtained by the sum of the ratings we obtain $\mu = 17,900$ This procedure is not strictly correct as a method of averaging, but it is useful as a check We believe that the scatter of computed values of μ is due entirely to random experimental errors in the method of measurement

If we accept the evidence of the mass plot in Fig 4, there is clear suggestion of a critical temperature at 15°, with change of μ, the slope for the high temperature portion is approximately $\mu = 12,000+$ The best fitting line in the lower temperature range (where the scatter is less) gives $\mu = 19,000$ — agreeing with the values found by Stier (1932–33) for O_2 utilization by yeast (3–15°), and by Lineweaver, Burk, and Horner (1931–32) for O_2 utilization by *Azotobacter* Stier found two critical temperatures, one of which for the strain used comes at $15 7 \pm 0 27°$ and another comes at 29° The values of μ are 19,530 cal below 15 7°, 12,440 cal from that temperature to 29°, and 8,290 cal above 29° The points are scattered within parallel bands the width of which is greater at higher temperature ranges It is of interest that for the same process in two different unicellular organisms the values of μ, and possibly the occurrence of a critical temperature should be so similar If our experiments were carried to higher temperatures it might be that more definite breaks would also be found for *Chlorella* (For a comparison of various values of μ reference is made to Stier's paper, 1932–33)

V

Emerson found in *Chlorella* an increase to about four times the rate of respiration when dextrose was added to the medium French, Kohn, and Tang (1934) find a rise to about twice at 18 5° and only 1 3 times at 3 5° The question arises as to what part the respiration in absence of added dextrose plays in the O_2 consumption measured in

glucose solution, and as to the correction which perhaps should be made so that the glucose oxidation may be analyzed alone The respiration in absence of added dextrose we may term normal The three possibilities are (1) that all the O_2 enzyme complex is used by the glucose as fast as it is formed, cutting out completely the "normal" process, (2) that the normal process goes on as usual while the glucose oxidation proceeds independently, utilizing another source of O , and (3) that both the normal and glucose processes compete for the same source of active O_2 in such a way that the normal rate is decreased

French, Kohn, and Tang (1934–35) have found that the normal respiration, if studied after removal to darkness for short periods corresponding to the times used in these experiments with added dextrose, gives a μ plot that has to be fitted with a curve or with two straight lines having quite different slopes with a critical temperature at about 11 5°, the temperature characteristics are respectively $\mu = 3,800$ and $\mu = 16,000$ above and below this critical temperature, the further analysis of this situation is considered in the succeeding paper, where it appears that the temperature characteristic for photosynthetically stored glucose alone is 19,500 (0 6–11 5°) and 3,500 (11 5–28°) If this normal process went on simultaneously with the glucose oxidation we should not expect a single straight line to fit the observations over this range We then conclude that the μ value here found, 19,000, refers only to the oxidation of the added glucose by the cells and is not in part dependent on the "normal" metabolism evident in the absence of glucose from the medium

SUMMARY

The temperature characteristic for the rate of O_2 consumption by *Chlorella pyrenoidosa* suspended in Knop solution containing 1 per cent glucose was studied between 1° and 27°C with the Warburg technic The value of μ was found to be about 19,000 ±1,000 cal There is some indication of a critical temperature at 20°C , with shift to a lower μ above this temperature The effect of sudden changes in temperature on the rate of respiration and the variation of the latter with time at constant temperatures are discussed It is concluded that the "normal" respiration (in absence of external glucose) does not appear in the determination of this temperature characteristic

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CONDUCTION IN NERVE FIBRES

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It is to be expected on general grounds that the conduction process and the excitation process in nerve will have common factors   This expectation has led to the assumption (1) that the nerve impulse may be propagated by means of the action potential which is thus required to stimulate succeeding inactive sections of the nerve in a manner quite analogous to that in which a potential applied to the outside excites a resting nerve at a certain region   Following this assumption one should be able to derive equations representing the conduction process from those which have already been established for the process of electrical excitation and thereby determine the common factors of the two processes   The validity of the original assumptions as well as that of the electrical excitation equations used in carrying through the analysis will be tested by data concerning both the velocity of the impulse and the electrical excitation of the same preparation at the same time

Equations of conduction have been derived by Rashevsky (2–4) on the above assumption using several types of excitation equations   Data on both the velocities of the impulses and time intensity curves of the same preparation at the same time have been given by E. A. Blair and Erlanger (5)   The present purpose is to discuss the consistency of these two developments

Rashevsky (2) first used the excitation equations of Hoorweg (6) and Lapicque (7), but neither of these gave a constant velocity of conduction   As this seems to be required (8) they will not be considered   Those of H. A. Blair (9) do, however, predict a constant velocity (3)

Blair's fundamental assumption is that the local excitatory process p grows according to the following equation

$$\frac{dp}{dt} = KV - kp \qquad (1)$$

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where K and k are constants, and V is the stimulating volta;
(3) leads to the following expression for the velocity, v, of the

$$v = \frac{(I - R)k}{R\alpha}$$

where I and R respectively, are the action current and the limi
of the action current necessary for excitation, k is as that of
1 and α is given by,

$$\alpha^2 = \frac{2(\gamma - 1)\rho}{\gamma \delta \bar{\rho} r}$$

where γ is the ratio of the resistance of unit length of the co
nerve fibre to that of unit length of the outside sheath, ρ and
specific resistivities of the core and the intermediate sheatl
tively δ the thickness of this sheath, and r the radius of the co

E A Blair and Erlanger (5) obtained voltage-capacity c
several fibres in the same trunk in several preparations as wo
velocity of the impulse in each   These data are given in '
II, and III   In Table I are the data for the voltage-capacit
of two preparations, two sets from each using different co
distances   The fibres are numbered starting with the fas
the columns give the voltages and capacities required to ex
in turn   The two rheobases are the initial and the final resp
In Table II are the similar data for a single set on a third prej
In Table III are tabulated the velocities in the fibres agai
numbers as in Tables I and II   The resistance of the condens
was in each case 26,000 ohms

According to integrals (9) of Equation 1 the voltage-capac
should conform to the equation,

$$\frac{R}{V} = \sigma k^{\frac{1}{1 - c r k}}$$

where V is the stimulating voltage, R the rheobase, c the cap
the condenser r the resistance of the circuit, and k as in Equ
Taking logs of both sides and arranging,

TABLE I

1st nerve long (125 mm) conduction distance temperature 21 2 C.

Fibre No	1	2	3	5		6	7	8
C	V	V	V	V	C	V	V	V
∞	0 197	0 302	0 55	0 815	∞	1 315	1 51	1 844
∞	0 215	0 303		0 835	1 09			3 11
1	0 215	0 347	0 649	0 9815	1	1 97	2 165	3 225
0 7				1 024	0 9	2 004		3 477
0 5	0 228	0 374	0 7	1 064	0 8		2 332	3 448
0 3				1 146	0 7	2 204		3 842
0 25	0 24	0 412	0 791		0 6		2 65	3 977
0 1	0 2747	0 46	0 919	1 441	0 5		3 065	4 322
0 075				1 546	0 4		3 417	4 724
0 05	0 318	0 506	1 035	1 667	0 3	3 35	4 287	5 76
0 03	0 384	0 589	1 204	1 901	0 2		5 415	7 694
0 02	0 453	0 696	1 403	2 181	0 1	6 374	12 134	
0 01	0 656	1 046	1 88	3 141	0 014	12 134		
0 007	0 830	1 198	2 299					
0 005	0 021	1 564	2 699	5 062				

1st nerve short (86 mm.) conduction distance temperature 21 2 C

Fibre No	1	2	3	4		6	7	8
C	V	V	V	V	C	V	V	V
∞	0 318	0 412	0 826	1 371	∞	2 228	2 756	3 488
∞	0 31	0 444	0 876	1 307	∞	2 077	2 7	
1 0	0 353	0 486	1 023	1 901	3 0		3 57	
0 5	0 368	0 522	1 112	2 186	1 0	3 499	5 53	5 78
0 25	0 403	0 554	1 207	2 288	0 9	3 622	5 67	5 956
0 1	0 476	0 649	1 415	2 675	0 8	3 87	5 916	6 067
0 08				2 739	0 7	4 059	6 412	6 68
0 05	0 542	0 741	1 576	2 915	0 6	4 205	6 63	7 136
0 03	0 637	0 848	1 79	3 865	0 5	4 562	7 22	8 073
0 02	0 754	1 012	2 078		0 4	5 023		9 392
0 01	1 050	1 373	2 765	4 741	0 34			10 732
0 007	1 162	1 694	3 34	6 067	0 3		9 0	
0 005	1 469	2 064	4 245	8 026	0 2	7 382	10 138	
					0 1	11 29	11 382	
					0 07		12 134	

Resistance 26,000 ohms
C, capacity in microfarads, V, volts

TABLE I—*Concluded*

2nd nerve short (63 mm) conduction distance temperature, 22°C

Fibre No	1	2	3	4		5
C	I	I	V	I	C	V
∞	0 161	0 178	0 237	0 33	∞	1 133
∞	0 189	0 188	0 235	0 331	∞	1 154
1 0	0 169	0 2	0 261	0 361	∞	1 137
0 5	0 18	0 213	0 265	0 377	1 0	1 571
0 1	0 238	0 268	0 338	0 473	0 7	1 687
0 08	0 25	0 275		0 499	0 5	1 817
0 07			0 355		0 3	2 040
0 06	0 276	0 301		0 554	0 2	2 112
0 05			0 393		0 1	2 737
0 04	0 326	0 35		0 591	0 08	2 826
0 03			0 478		0 05	3 266
0 02	0 456	0 472		0 66	0 02	4 613
0 01	0 698	0 687	0 736	1 112	0 004	10 004
0 008	0 824	0 805	0 962			
0 007				1 383		
0 005	1 15	1 127	1 43			
0 002	2 622	2 591	2 995	3 85		

2nd nerve long (101 mm) conduction distance, temperature, 22°C

Fib e No	1	2	3		4	5
C	I	I	V	C	V	V
∞	0 069	0 0805	0 126	∞	0 261	0 41
∞	0 072	0 0805	0 132	∞	0 261	0 408
1 0	0 079	0 0882	0 135	1 0	0 2967	0 468
0 5	0 0805	0 0912	0 1365	0 5	0 3083	0 58
0 1	0 105	0 1165	0 1642	0 1	0 359	0 723
0 05	0 1242	0 1365	0 1885	0 08		0 729
0 04	0 135	0 1504	0 201	0 06		0 787
0 03	0 155	0 1665	0 2192	0 05	0 397	0 8125
0 02	0 1895	0 197	0 2585	0 04	0 412	0 847
0 01	0 276	0 287	0 366	0 03	0 447	0 915
0 008			0 439	0 02	0 509	
0 005	0 442	0 455	0 582	0 01	0 7	1 396
0 002	0 985	0 993	1 293	0 008	0 791	1 571
				0 005	1 069	2 136
				0 002	2 334	4 682

TABLE II

	3rd nerve							
Fibre No	1 k = 2250 v = 2570		2 k = 2650 v = 2360		3 k = 3100 v = 1750		4 k = 3100 v = 1750	
C	V obs	V cal	V obs	V cal	V obs.	V cal.	V obs	V cal.
∞	0 109	0 109	0 132	0 132	0 215	0 215	0 30	0 310
∞	0 11		0 135			0 215	0 311	
1 0	0 1092	0 117	0 148	0 140	0 235	0 222	0 332	0 318
0 5	0 1186	0 123	0 153	0 147	0 241	0 236	0 356	0 34
0 1	0 154	0 157	0 19	0 183	0 284	0 289	0 442	0 415
0 05	0 186	0 191	0 221	0 219	0 323	0 342	0 493	0 52
0 025	0 246	0 247	0 288	0 279	0 532	0 429	0 601	0 617
0 01	0 392	0 397	0 448	0 436	0 612	0 653	0 902	0 94
0 008	0 458	0 455	0 49	0 501	0 697	0 740	1 027	1 06
0 006	0 576	0 547	0 591	0 595	0 865	0 880	1 25	1 27
0 004	0 716	0 727	0 749	0 782	1 103	1 14	1 614	1 65
0 002	1 377	1 205	1 52	1 32	2 06	1 89	2 97	2 73
0 001	2 23	2 22	2 41	2 34	3 4	3 34	5 14	4 81

Fibre No	6 k = 1900 v = 621		7 k = 225 v = 201	
C	V obs	V cal	C	V obs	V cal.
∞	0 928	0 928	∞	1 86	1 86
∞	0 909		∞	1 52	
1 0	1 027	1 03	1 0	2 063	1 93
0 5	1 1	1 04	0 7	2 73	2 40
0 1	1 41	1 40	0 5	3 167	3 25
0 05	1 58	1 72	0 3	3 94	3 91
0 025	1 91	2 28	0 2	4 86	4 68
0 01	2 71	3 70	0 1	7 93	6 78
0 008	3 065	4 30	0 075	8 34	7 45
0 006	3 75	5 22			
0 004	4 81	7 00			
0 002	12 0	12 05			

Resistance 26,000 ohms

C, capacity in microfarads, V obs , the observed strength of the stimulus in volts, V cal , the voltage calculated from Equation 4 v the velocity in centimeters per second, k as in Equation 4

TABLE III

	Fibre No	v	R'	k	k/R'	log k/R'	log v
1st nerve long	1	2,160	0 206	2,520	12,200	4 086	3 335
	2	1,740	0 302	2,600	8,609	3 935	3 241
	3	1,160	0 55	2,450	4,454	3 649	3 065
	5	567	0 825	1,560	1,891	3 276	3 754
	6	199	1 315	220	167	2 223	2 299
	7	130	1 51	128	84 7	1 928	2 114
	8	65 8	1 844	97	52 6	1 721	1 818
1st nerve short	1	1,686	0 315	2,600	8,220	3 915	3 227
	2	1,560	0 428	2,450	5,720	3 757	3 193
	3	1,090	0 855	2,410	2,825	3 451	3 037
	4	574	1 339	1,900	1,421	3 153	2 759
	6	210	2 152	152	70 2	1 846	2 322
	7	79	2 728	215	78 8	1 896	1 898
	8	45 5	3 488	150	43 0	1 634	1 659
2nd nerve short	1	1,570	0 161	1,620	10,030	4 001	3 196
	2	1,430	0 184	1,955	10,630	4 027	3 155
	3	1,370	0 236	2,170	9,170	3 962	3 137
	4	1,015	0 330	2,215	6,710	3 827	3 006
	5	332	1 140	585	513	2 710	2 521
2nd nerve long	1	1,740	0 070	1,930	27,570	4 440	3 241
	2	1 740	0 0805	2,270	28,200	4 450	3 241
	3	1,330	0 129	3,650	28,300	4 452	3 124
	4	927	0 261	3,300	12,650	4 102	2 967
	5	423	0 409	1,790	4,380	3 641	2 626
3rd nerve	1	2,570	0 109	2,250	20,600	4 314	3 410
	2	2,360	0 133	2,650	19,900	4 299	3 373
	3	1,750	0 215	3,100	14,400	4 158	3 243
	4	1,750	0 305	3,100	10,150	4 006	3 243
	6	621	0 920	1,900	2,070	3 316	2 793
	7	201	1 86	225	121	2 083	2 303

v, the velocity in centimeters per second, R', the observed rheobase voltage, k as in Equation 4

A means of obtaining the k of each fibre is thus provided This is conveniently done by plotting $cr \log \dfrac{V}{R}$ as ordinates against $\log cr +$
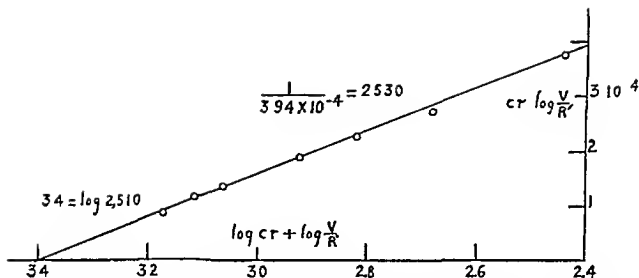
FIG 1 The data of the fastest fibre, No 1, of the first nerve long plotted according to Equation 5
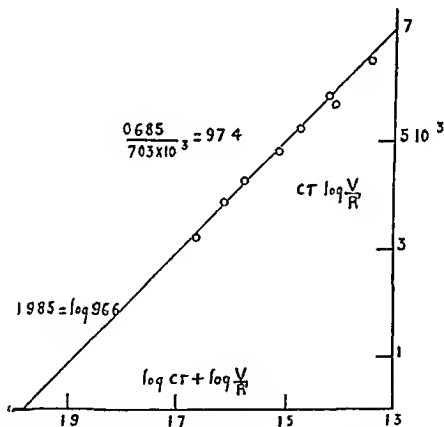

FIG 2 The data of the slowest fibre No 8 of the first nerve long plotted according to Equation 5

$\log \dfrac{V}{R}$ as abscissae in each case   The slope of the resulting straight line gives $1/k$ and its intercept on the axis of abscissae gives $\log 1/k$

It is found that the data of Erlanger and E A Blair conform quite

well to this relation   Illustrative examples are given by plotting in Figs 1 and 2 in this fashion the data of the fibres with the greatest and the least velocity respectively of the first set of data in Table I Also the whole of the data of the third preparation are given in Table II with measured and calculated voltages, the latter having been obtained by assuming the capacities, resistances, and rheobases to be correct as given and by taking the k values derived from such repre-
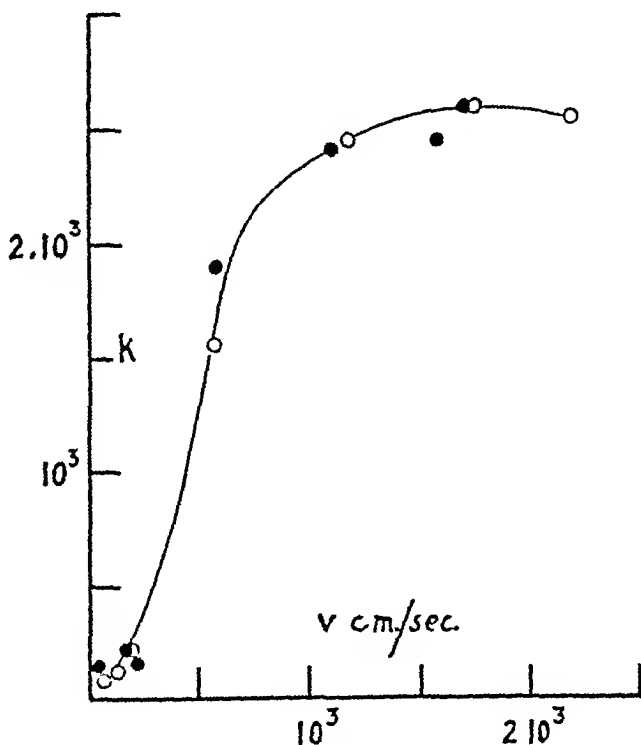

FIG 3   The k of each fibre against its velocity for the first nerve   Long preparation, circles, short, dots

sentations as Figs 1 and 2   It will be seen that the agreement of the data with Equation 4 is very good throughout except for Fibre 6 In the whole set there were a few cases such as this in which the voltage-capacity curve seemed to be mixed somewhat, i e , it seemed to correspond in various parts to more than one fibre   Apart from these few cases however, the use of Equation 4 as a criterion shows that the voltage-capacity curves of the separate fibres in a trunk can be ob-

tained, by E A Blair and Erlanger at least, with about the same clearness and precision as is obtainable on the most excitable group of fibres in a trunk in the ordinary nerve muscle preparation
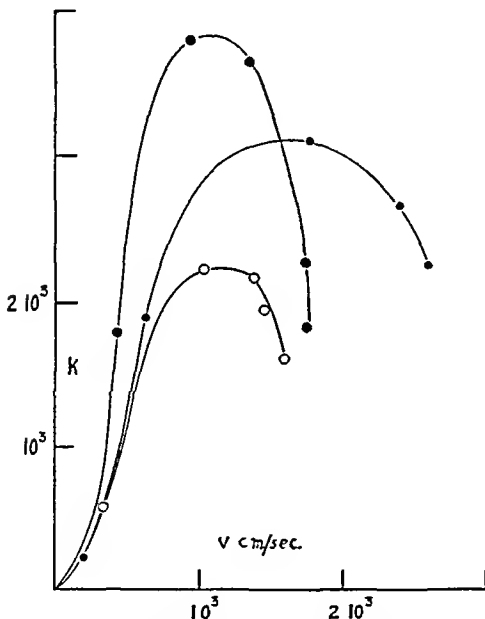
FIG 4 The k of each fibre against its velocity for the second and third nerves Second short, circles, second long, large dots, third, small dots

In Figs 3 and 4 are plotted the resulting k values against the veloci ties of the impulses, the first figure being for both experiments on the first nerve and the second for the second and third nerves The curves show that the velocity is not a linear function of the excitability as has been suggested previously (7) A given excitability may in fact

have two corresponding velocities   The smoothness and the regu-
larity of the curves do indicate, however that the velocity is a func-
tion of k The nature of this function will be discussed later

The values of k and c may now be related to Equation 2   This
equation presents at once the difficulty that it involves the quantities
I R, and α which have not been measured   The quantity α since it is
a function of the radius alone in fibres of similar structure and con-
stituents, is perhaps measurable with fair accuracy on isolated fibres
but in general it will probably be impossible to obtain its value with
the present type of experiment so that it must be dealt with indirectly
The quantity $(I - R)/R$ can perhaps be measured directly through the
following circumstance   At the moment an external stimulus becomes
adequate the action current commences to flow at the excited region
and the unexcited region immediately adjoining is subject to the
maximum value of this current. i e , it is subject to a constant current
and it should be excited according to the integral[1] of Equation 1 for
direct current (9),

$$t_1 = \frac{1}{k} \log \frac{I}{I - R} \tag{6}$$

That is the impulse will not start to travel down the nerve until after
an interval t_1 following the moment at which the external stimulus
becomes adequate   This lag has been in fact observed (10, 5) but
not measured in the present instance for each velocity   E A Blair
and Erlanger (5, p 530) give as extreme values $t_1 = 0\,25\ \sigma$ approxi-
mately for the high velocity fibres and $2\,4\ \sigma$ for the low   From Table I
the i of the most irritable fibre is probably about 2500, of the least
about 100   Therefore in the first case using Equation 6,

$$\frac{0\,25}{100} = \frac{1}{2500} \log_e \frac{I}{I - r}$$

these values are extremes it is indicated that $(I - R)/R$ is about the same for all velocities but it may increase as the velocity decreases It is perhaps more likely on general grounds that the action current exceeds the rheobase by an amount approximately constant, $i\,e$, that $I - R$ is constant, rather than $(I - R)/R$

If then for the present I-R is taken to be constant, Equation 2 may be written,

$$v = \text{constant}\,\frac{k}{\alpha R} \tag{7}$$

But it is desirable to express R in terms of the external rheobase, R', which is contained in the data   Since the voltage drop per unit length of trunk varies as R' the current in any particular fibre will vary as R' But the relative currents in different fibres will vary conjointly with their quantities α and their conductivities, $i\,e$, as $\alpha\,r$ Therefore R varies as $\alpha\,r^2 R'$ and if γ and ρ are constants for all fibres Equation 7 becomes

$$v = \text{constant}\,\frac{k}{\alpha^2 r^3 R'} \tag{8}$$

But since α varies as $1/r$ this may be written,

$$v = \text{constant}\,\frac{k}{rR} \tag{9}$$

It is usually assumed on experimental grounds that v varies as r to a small power, so that it will be permissible to write $v = r^q$ or $r = v^{\frac{1}{q}}$ so that from Equation 9

$$v^{1 + \frac{1}{q}} = \text{constant}\,\frac{k}{R} \tag{10}$$

or taking logarithms,

$$\left(1 + \frac{1}{q}\right)\log v = \log\frac{k}{R} + \text{constant} \tag{11}$$

In Fig 5 are plotted these logarithms for both of the experiments

on the first preparation and in Fig 6 those for the other two preparations   The numerical equation of Fig 5 as it is drawn is,

$$1\ 59 \log v = \log \frac{k}{R'} + 1\ 22$$

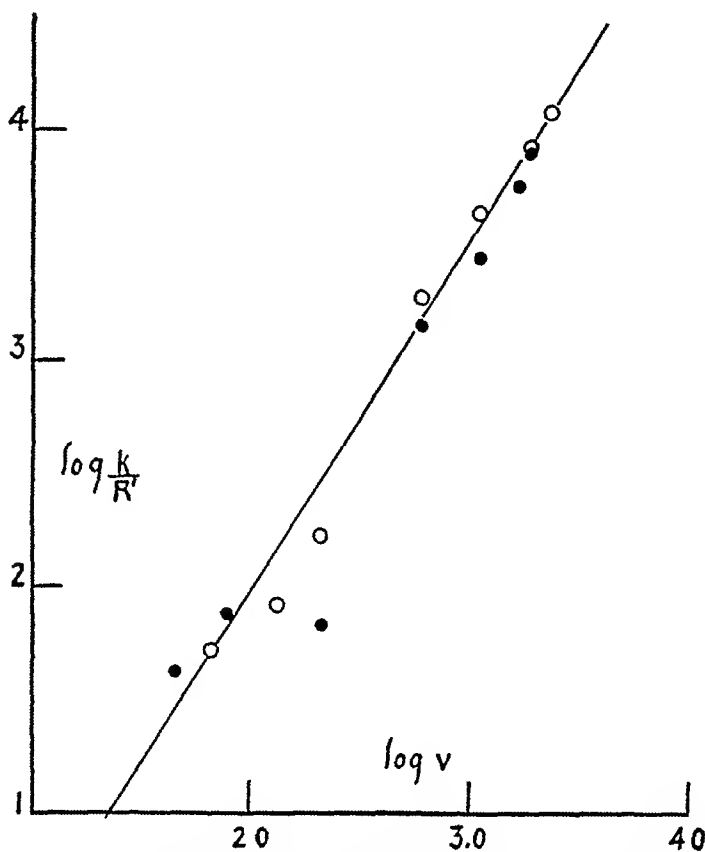so that $1 + \dfrac{1}{q} = \dfrac{3}{2}$ approximately, or $q = 2$ The lines in Fig 6 are

FIG 5   Log k/R' with log velocity for the first nerve   Long, circles, short, dots

drawn with the slopes 1 5 exactly corresponding to $q = 2$ exactly

It will be seen that the data agree with the Equation 11 in rough approximation   The agreement probably is not as good as can be expected from the accuracy of the data but it may not be proper to assume $I - R$ as constant nor proper to assume that all the fibres are similarly constituted, nor that the velocity varies exactly as a power of the radius   All these things are probably approximately true, how-

ever, so that the agreement obtained can be taken as an indication of the validity of Rashevsky's analysis

The finding $q = 2$ above leads to the conclusion that the velocity varies as the square of the radius The lines of Figs 5 and 6, however, might equally well have been drawn with slopes 5/3, in which case would $1/q = 2/3$ so that the velocity would vary as the radius to the
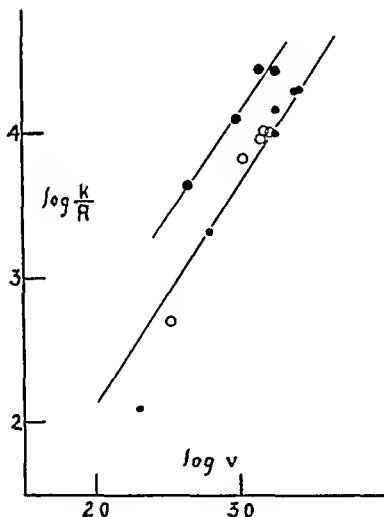
FIG 6 Log k/R' with log velocity for the second and third nerves Second short circles, second long, large dots, third, small dots

power 3/2 In either case the conclusion reached here is consistent with the conclusion from experiment that the velocity varies as a small power of the radius (5), and this is in further support of the validity of the analysis More exact conclusions can be drawn when data have been obtained giving $(I - R)/R$ along with v and k, and when it has been better determined how the velocity and radius are related

Two other relations are given in Figs 7 to 10   In Figs 7 and 8 respectively are plotted log v and R' for the first preparation and for the other two   These data conform quite well to the relation,

$$\log v = -a R' + \log b \tag{12}$$
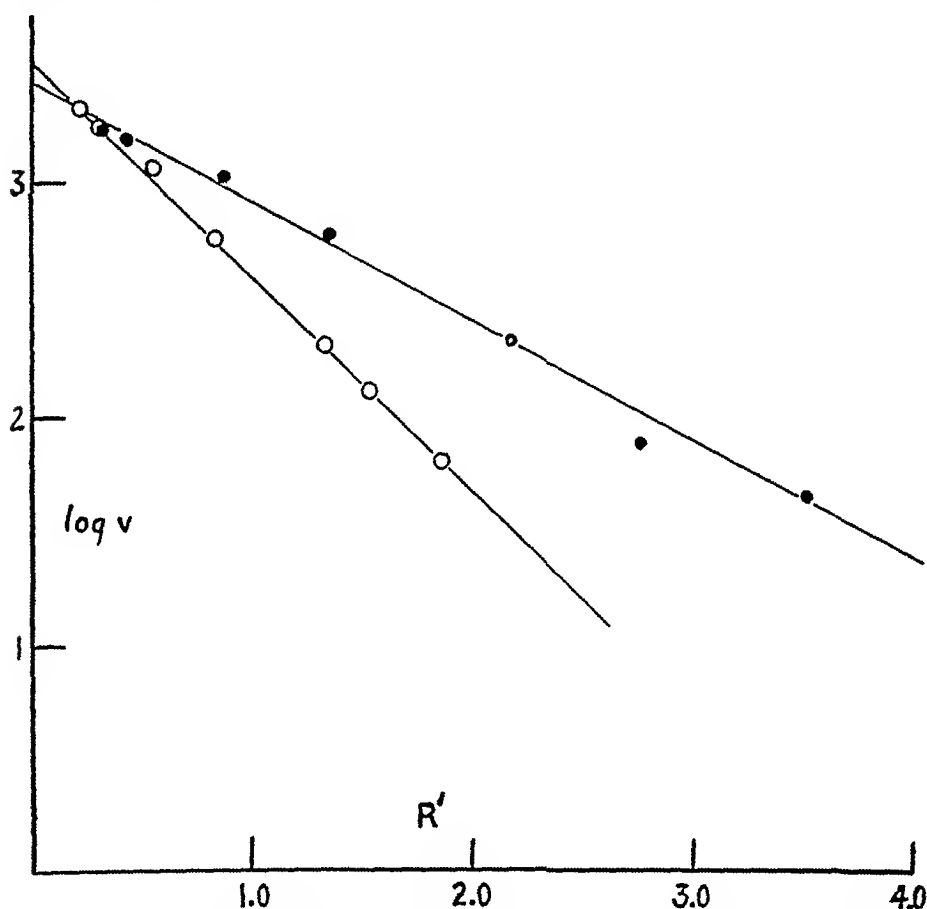
where a and b are constants

Fig 7   Log velocity with the rheobase for the first nerve   Long, circles, short, dots

As a consequence of Equations 11 and 12 must

$$\log \frac{k}{R'} = -cR' + \log d \tag{13}$$

where c and d are constants   The data of the first preparations are plotted for this relation in Fig 9 and those for the second and third preparations in Fig 10

From Equations 12 and 13 it is possible to write the equation for the relation of k to v in Figs 2 and 3 which is,

$$k = \text{constant } v^{\overline{a}} \log \frac{b}{v}$$

This equation expresses the empirical relation of the velocity to the excitability as it appears from the present data

It will be noted in the data of Table III that with the second nerve (long) there is a single velocity 1740 in two fibres of different k's   This
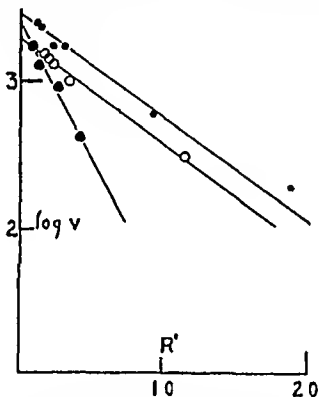
Fig 8 Log velocity with the rheobase for the second and third nerves   Second short, circles, second long, large dots, third, small dots

is understandable on reference to Fig 2 because the points lie on a very steep part of the curve where k changes very rapidly as the velocity changes slowly   With the third nerve there is a case where both the velocities and the k's are equal   These are represented by the single uppermost point of the middle curve of Fig 2   At this region one might expect very different velocities with k's approximately equal This expectation is not realized in the middle curve but in the lower curve, second nerve short, are the points $k = 2170$, $\neg = 1370$, $k = 2215$, $v = 1015$ These k's are approximately equal but the velocities

are greatly different　These illustrate the point that in certain regions
greatly different velocities can obtain with excitabilities of about equal
values while in other regions velocities approximately equal may exist
with greatly different excitabilities
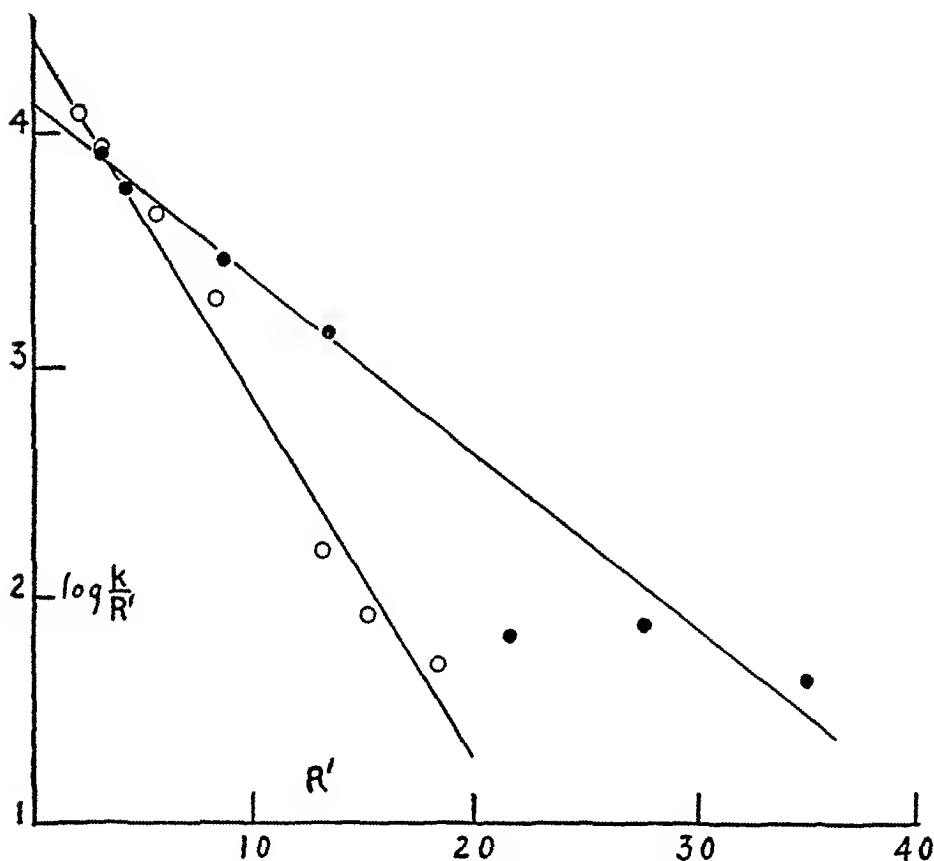
FIG 9　Log k/R' against the rheobase for the first nerve　Long, circles, short,
dots

The empirical relation of Equation 12 is much the simplest of the
relations of the velocity to stimulation data　It indicates that the
right hand part of Equation 2 can be expressed in terms of the rheobase
alone but it is scarcely worth while to attempt this until data have
been obtained on the quantity $(I - R)/R$ at least

In conclusion it appears that the data of E　A　Blair and Erlanger
lend support to Rashevsky's formulation of the idea that the nervous
impulse is propagated by means of the action current, which excites

successive regions according to H A Blair's excitation equations   In
any case it is established that voltage-capacity curves conforming to
the usual criteria are obtainable on the separate fibres of a trunk
The rheobases and excitabilities as derived from these curves conform
to comparatively simple empirical relations with the velocity   This
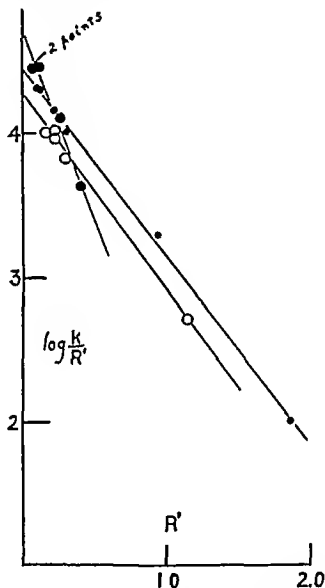
Fig 10 Log k/R' against the rheobase for the second and third nerves Second
short, circles, second long large dots third, small dots

indicates that the velocity is a function of the same quantities of
which the voltage capacity curves are a function   Although such
functional relationships between transmission and excitation are pos
sible if both processes, even though they are quite different, depend
on the dimensions of the tissue elements, their existence does not

appear to be at all probable unless the processes are of the same kind. Therefore, even though the present theoretical views are not substantiated by further investigation, there is good reason to believe that the basic hypothesis that the action current is the primary factor in transmission is correct

No mention has been made of the possibility of the exciting current acting only through the nodes of Ranvier (12, 1) Rashevsky (3) has analyzed this case also   The velocity under certain conditions approximates the value given by Equation 2 but this problem cannot be considered further from the present point of view without a knowledge of the internodal distances involved in each case

SUMMARY

Data by E A - Blair and Erlanger on the voltage-capacity curves and the nerve impulse velocities of each of several fibres in the same nerve trunk are related to Rashevsky's equation for the velocity of transmission in nerve   The results lend support to Rashevsky's analysis   Other empirical relations between the velocity and the parameters of the excitation equations indicate the correctness of the hypothesis that the action current is the primary factor in transmission, which process is carried on by the electrical excitation of successive regions of the nerve fibre by means of its action current according to the ordinary laws of electrical excitation

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THE PREPARATION OF THE GRADED COLLODION MEM-
BRANES OF ELFORD AND THEIR USE IN THE
STUDY OF FILTERABLE VIRUSES

By JOHANNES H. BAUER and THOMAS P. HUGHES

(*From the Laboratories of the International Health Division of the Rockefeller Founda
tion, New York*)

During the past 50 years many attempts have been made to develop
a method for preparing permeable collodion membranes of graded
and uniform pore size   Such membranes would constitute an exceed
ingly important apparatus in biological investigation and should be
especially useful in the study of filterable viruses

The method of preparation which has been most extensively used is that of
Bechhold (1–3), consisting of impregnating filter paper with solutions of nitrocel
lulose in glacial acetic acid, the permeability being regulated by the concentration
of nitrocellulose used   These membranes have the serious defect of considerable
variability in the size of pores Elford (4) estimates that the largest pores in such
a membrane may have a radius ten to twenty times greater than the average pore
radius

A number of investigators have attempted to devise a method by which the
use of filter paper could be eliminated and strong membranes of graded and uniform
porosity be prepared   Bigelow (5) Walpole (6), Bartell and Carpenter (7),
Hitchcock (8), Pierce (9) and others have controlled the permeability of mem
branes by varying the time allowed for evaporation of solvents from an ether
alcohol solution of collodion   Eggerth (10) graded the membrane permeability
by varying the alcohol content of the collodion solution   Brown (11 12) and
also Nelson and Morgan (13) treated air-dried collodion membranes with different
concentrations of alcohol in water and so effected variation in permeability
Among non volatile reagents added to ether alcohol solutions of collodion for the
purpose of altering membrane permeability are ethylene glycol (Pierce (9)) and
glycerol (Schoep (14))   Asbesbov (15) was the first to utilize volatile reagents,
adding varying amounts of acetone to increase or amyl alcohol to decrease mem
brane permeability   In later work he recommends the use of acetic and formic
ethers (16) and states that the results obtained by adding amyl alcohol are
unreliable

Elford (4), using the same ingredients as Ashesbov, found that acetone and

amyl alcohol are mutually "antagonistic" in their solvent action on nitrocellulose
While either one in conjunction with alcohol and ether is an excellent solvent, the
presence of both, combined in certain concentrations, results in coagulation of
nitrocellulose   Consequently he prepared a concentrated solution of nitrocellulose
in ether and alcohol and to this added certain fixed proportions of amyl alcohol
and acetone   When this solution was poured on glass plates and the solvents were
allowed to evaporate, the amyl alcohol, being the least volatile, increased in relative
concentration and in the presence of acetone initiated an aggregation of the nitro-
cellulose, while the residual ether and alcohol maintained a spontaneous gelling
process   The resulting membranes were highly permeable and had considerable
tensile strength   Elford also found that the addition of small amounts of glacial
acetic acid to the collodion solution decreased the permeability of the membranes,
while the addition of water increased it   Utilizing these findings he devised a
very satisfactory method by which finely graded membranes can be prepared,
having adequate tensile strength, and ranging in size from 10 mμ to 3 μ in average
pore diameter

A need of membranes suitable for ultrafiltration has been felt in this laboratory
for some time   After a review of the various methods recommended for the prepa-
ration of graded collodion membranes, Elford's method appeared to be the most
practical   Consequently it was selected for study

<div align="center">EXPERIMENTAL</div>

Preparation of Stock Collodion

A small supply of Necol, the collodion preparation recommended by Elford, was
secured from England and was used in early experiments   Considerable difficulty
was encountered at the beginning in attempts to prepare membranes similar to
those he described   Although his technique was followed as closely as possible,
it was found impossible to produce membranes of a similar quality on successive
attempts   Occasionally membranes satisfactory in all properties were obtained,
however, using the same collodion mixture and duplicating the procedure as closely
as possible, membranes of an entirely different quality would result   After some
experimentation and a study of all the factors involved, we eventually succeeded
in preparing consistently satisfactory membranes from Necol, and thus confirmed
Elford's results

Necol cannot be purchased in this country, and when our original supply be-
came exhausted a new lot was ordered from England   After considerable delay,
it was supplied to us from the firm of Du Pont De Nemours, of Parlin, New Jersey,
to which our order apparently had been referred   Their product was labelled
Collodion X-660-18 and showed the same consistency and appearance as Necol
Membranes made from it were similar in quality to those made from Necol

Although membranes made from Necol or Collodion X-660-18 were quite
satisfactory as far as graded permeability was concerned, their thickness was

excessive ranging from 0 2 mm to 0 3 mm   As thinner membranes are desirable Collodion ⅃ 660 18 was diluted with one half its weight of a solvent consisting of 75 per cent anhydrous ether and 25 per cent absolute alcohol by weight   This dilution was made before any acetone or amyl alcohol was added   The results were unsatisfactory since the membranes obtained were very friable and were difficult to remove from the plates   Also their porosity could not be regulated by the addition of glacial acetic acid

Inasmuch as the identity and proportions of the solvents used in commercial collodion preparations such as Necol and Collodion ⅃ 660-18 are unknown to us, it seemed advantageous to prepare a stock collodion of known chemical constitution which could be diluted if desired without danger of disturbing the effective equilibrium between ingredients   After some experimentation a collodion was prepared which was found to give consistently good results   The most convenient form of nitrocellulose was found in Du Pont s parlodion which is sold in shreds immersed in water   The solvent selected consisted of 75 per cent anhydrous ether and 25 per cent absolute alcohol by weight   The exact composition of the collodion is as follows

Parlodion shreds	150 gm
Absolute alcohol	250
Anhydrous ether	750
Acetone	1150
Amyl alcohol (primary)	575 cc

The parlodion shreds are washed six times in distilled water twice in 95 per cent alcohol and twice in absolute alcohol   They are not dried after washing   In early experiments dried shreds were used   The resulting solutions were quite turbid and on standing showed a quantity of precipitate   Membranes made from these solutions were found to be less permeable than if drying had been omitted

In our early experiments the chemicals used for solvents were subjected to additional purification as recommended by Elford (4)   It was later found that untreated chemicals of the analytical reagent grade gave equally satisfactory results   consequently the additional purification was thereafter omitted

After the final washing 250 gm (316 cc) of absolute alcohol freshly prepared by drying 95 per cent alcohol over lime and distilling is added to the parlodion and the shreds allowed to swell overnight   On the following day 750 gm (1043 cc) of anhydrous ether is added and the mixture shaken at intervals until the parlodion is completely dissolved   The solution is then diluted with its own weight of acetone (1453 cc) and agitated in a mechanical shaker for 2 hours   Primary amyl alcohol is then added at the rate of 10 cc to each 40 gm of the mixture (575 cc) and the solution is again shaken for 2 hours   This stock solution must be allowed to stand 2 to 3 weeks before use   To date seven different solutions have been prepared by this method and all have given uniformly satisfactory results

It will be noticed that acetone and amyl alcohol are used in the same proportion as recommended by Elford (4)

Recently we obtained a supply of parlodion manufactured by the Mallinckrodt Chemical Works   This is sold in the dry state although the shreds have the same appearance as the Du Pont product   A solution was prepared using this product as the source of nitrocellulose and following the above mentioned procedure, with the exception that the washing of the parlodion was omitted   The resulting collodion was water-clear, and the membranes prepared from it were of excellent quality

Preparation of Membranes

The apparatus used in the preparation of membranes is similar to that described by Elford and is illustrated in Fig 1   For routine purposes the membranes are cast in glass cells 40 cm in diameter   The cell is made from two pieces of plate
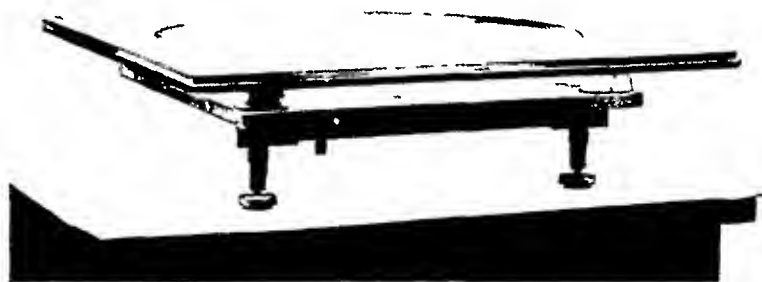
FIG 1   Cell and stand for preparation of membranes

glass, each 50 cm square and 7 mm thick   A circular hole 40 cm in diameter is cut in the center of one plate and the two plates are cemented together with egg white

The levelling table for supporting the cell is made of a piece of plate glass 15 inches square and 5/8 inch thick   This is fastened to a heavy metal triangle supported at one corner by a solid leg and at the remaining two corners by adjustable screws   To insulate the cell from the levelling table and to allow a free air circulation between them, four large rubber stoppers are placed at the extreme corners of the levelling table and on them the cell rests   If the cell lies directly on the levelling table, its temperature becomes uneven during the evaporation process, owing to retention of heat by the metal triangle   This in turn results in uneven permeability in the membrane   The cell is carefully levelled by aid of the adjustable screws, making observations with two sensitive spirit levels placed at right angles, thus insuring against unequal distribution of the collodion

The stock collodion solution is diluted with an equal volume of a diluent con-

sisting of one part by weight of absolute alcohol to nine parts by weight of an hydrous ether The diluted solution is measured in 200 cc amounts into a series of 6 ounce bottles having screw caps lined with tin foil Membranes prepared from this solution will have an average pore diameter of 0 6 to 0 8μ, depending on the humidity and temperature at which the solvents are allowed to evaporate If more, or less, permeable membranes are desired water or glacial acetic acid is added to the solution The addition of 2 cc. of water to 200 cc. of the diluted collodion gave membranes with an average pore size of 1 0 to 1 2μ If larger quantities of water are added considerable precipitate is formed and the resulting membranes have but little tensile strength When membranes of less than 0 5μ average pore size are desired, glacial acetic acid (analytical reagent grade) is added in amounts varying from 0 2 to 3 6 cc for each 200 cc. of diluted collodion As indicated above, conditions other than the chemical constitution of the diluent have considerable influence on the membrane permeability therefore it is impossi ble to establish a fixed scale of the exact quantities of ingredients necessary to produce a membrane of a given pore size In our experience, 1 cc of glacial acetic acid added to 200 cc of the diluted collodion solution, reduced the average pore size to 200 mμ The addition of 2 cc reduced it to 100 mμ, while the addition of 3 cc. reduced it to 15 mμ

After the addition of the required amount of acetic acid or water, the bottles are placed in a shaking machine and agitated for 2 hours The collodion solution is then carefully poured into the center of the levelled cell, where it quickly spreads over the entire surface The contents of one bottle (200 cc.) is used for each 40 cm cell The optimum evaporation time was found to be 75 minutes If this time is prolonged the membranes become less permeable and the surface becomes corrugated Shorter periods of time yield rather thick membranes of low tensile strength and spongy consistency The temperatures during the evaporation ranged from 22–24 C , and the relative humidity from 60 to 65 per cent

The membranes are prepared in a room measuring 12 by 24 feet with a ceiling 11 feet high The temperature is regulated by opening or closing the steam radia tors The desired humidity is obtained by running hot water from the faucets The doors and windows must be kept shut as it was found that air movement over the cell during the evaporation time resulted in the formation of a membrane one part of which was more permeable than the other During the summer months when the outdoor temperature exceeded 24 C , no satisfactory membranes could be prepared To assure a fair degree of reproducibility in membrane characteristics both the temperature and humidity must be kept constant Since the tempera ture of the collodion solution falls considerably below that of the room during the evaporation process moisture from the air is condensed into the collodion solution This absorbed water increases the coagulation of the nitrocellulose, and therefore, increased atmospheric humidity will result in increased membrane permeability Likewise drier air will cause decreased permeability

After the collodion solution has evaporated for the standard length of time, the

cell containing the membrane is submersed in tap water in a tank measuring 55 by 55 by 15 cm   The membrane separates itself completely from the glass after 5 to 10 minutes immersion   The membranes are then washed for periods of 2 to 3 weeks in distilled water   For this purpose photographic trays measuring 45 by 50 cm are used   Six membranes, separated from each other by large filter papers, are placed in each tray   The distilled water is changed daily

When the washing is completed, discs are cut from the membranes by means of a steel punch   The portion of the membrane within 3 cm from the edge is discarded   Our standard size disc used for ultrafiltration measures 38 mm in diameter   Consequently one large membrane yields 40 to 45 small discs   These are stored under water in Atlas E-Z seal fruit jars without the addition of preservative   The membranes will keep well in distilled water for a number of months   It was found, however, that on standing some membranes shrink considerably and become less permeable, while others remain unchanged for a period of 6 months or longer   It is therefore advisable to recalibrate membranes which have been stored for a month or more before using them for filtration experiments

Calibration of Membranes

The average pore sizes of the membranes are calculated by an application of Poiseuille's law, using data obtained by measurement of the thickness of the membrane, the amount of water passing through a measured area in a given time and under a known hydrostatic pressure, and the volume of pores in the membrane as shown by differences between its wet and dry weight   This law was derived to express the rate of flow through one capillary tube of a uniform diameter and was first applied to the calculation of pore diameters by Guérout (17), who tested the size of the pores in bladder, gold beater's skin, and parchment   Since then it has been applied to the determination of the size of pores in collodion membranes by Hitchcock (18), Bjerrum and Manegold (19), Elford (20), and Cox and Hyde (21), among others   The legitimacy of its application to this purpose has been investigated by Bigelow (5) and by Duclaux and Errera (22)   Both investigations indicated that the passage of fluids through collodion membranes is controlled by the same laws which determine the passage of liquids through capillary tubes

Calculation of Average Pore Size

Poiseuille's law governing the passage of water through a capillary tube is stated as

(1)
$$v = \frac{\pi p r^4}{8l\eta} t$$

where V is the volume of water passing in the time t under a pressure p, r the radius of the capillary, l the length of the capillary, and η the coefficient of viscosity of water at the temperature used

To apply the law of Poiseuille to the measurement of the average pore size of membranes, it becomes necessary to assume that the membrane represents a bundle of capillaries oriented at right angles to the membrane surface and equal in length to the thickness of the membrane   To compute the number of pores the following calculations may be made   If l represents a length of the capillaries (equivalent to the thickness of the membrane), then the volume of each capillary is $\pi r^2 l$ If W^2 represents the weight of the membrane when wet, and W^1 represents the weight of the same membrane when dry, the differences designated as W must represent the volume of pores Consequently $\frac{W}{l}$ must represent the total cross section area of all the pores, and since the area is πr, $\frac{W}{\pi r^2 l}$ represents the number of capillaries   Now if V is the amount of water passed through the membrane in the time t, then $\frac{V}{\frac{W}{\pi r l}}$ will represent the volume of water passed through one capillary, or the sum of the volumes may be expressed by Poiseuille's law as given in Equation 1   Transposing this formula to solve for r we obtain

(2)
$$r^2 = \frac{8l^2 \eta v}{w p t}$$

or, simplifying,

(3)
$$r = 2l \sqrt{\frac{2\eta v}{w p t}}$$

Equation 3 is the one used for determining the average pore size of membranes r, the radius of the average pore, and l the thickness of the membrane, are expressed in centimeters   Since the measurement of rate of flow of water is done at room temperature, η, the coefficient

of viscosity of water, is taken as 0 00893 V, the volume of water passing through the membrane is expressed in cubic centimeters, and t, the time required for this passage, is expressed in seconds W, the difference between the wet and dry weight, is expressed in grams and must be corrected to represent an area of 1 sq cm   The value V must be similarly corrected p, the average pressure producing the flow, is expressed in dynes, and since 1 cm water pressure is equivalent to 980 6 dynes, the value becomes 980 6 $\times$ average pressure in centimeters of water   The final formula then becomes

$$(4) \qquad r = 2l \sqrt{\frac{2\left(\begin{array}{c}\text{Coefficient of viscosity}\\ \text{of water at } 25^\circ\end{array}\right)\left(\dfrac{V}{2\pi \,(\text{radius of disc})^2}\right)}{\left(\begin{array}{c}980\ 6\ (\text{Average pressure in}\\ \text{centimeters of water})\end{array}\right)t\left(\dfrac{\text{Wet weight} - \text{dry weight}}{2\pi\,(\text{radius of disc})^2}\right)}}$$

Assigning concrete values, where the rate of flow was measured through an area of membrane 1 575 cm in diameter and the difference in wet and dry weight was determined for a membrane 3 15 cm in diameter, the final formula is

$$(5) \qquad r = \frac{2}{10}\sqrt{\frac{2 \times 0\ 00893}{\left(\dfrac{1\ 575}{3\ 15}\right)^2}\ \frac{1}{980\ 6}}\ l\ \sqrt{\frac{v}{wpt}}$$

Since the values in the first part of this equation are fixed, they can be represented by a constant K, and the working formula simplified to

$$(6) \qquad r = Kl\sqrt{\frac{v}{wpt}}$$

K, constant for fixed condition of measurements
l, thickness in millimeters
v, volume of water passed through membrane in cubic centimeters
W, difference in weight in grams
p, average hydrostatic pressure in centimeters of water
t, time in seconds

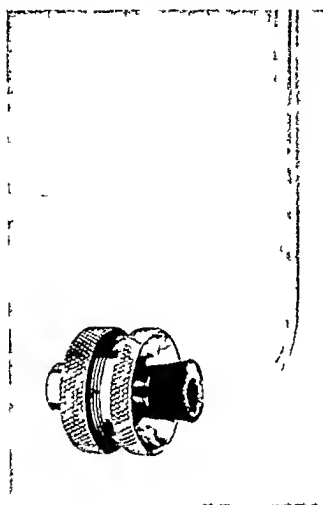Logarithms are most conveniently used in all computations

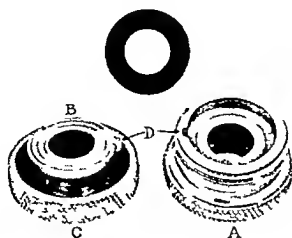Fig 2 Instrument used for determining the rate of flow of water

Fig 3 Instrument used for determining the rate of flow of water showing
details

Determination of Average Pore Size

To obtain the data for calculations, three determinations must be made on each membrane, the thickness, the rate of flow of water, and the difference between wet and dry weights

The thickness is measured by use of a micrometer caliper, such as is used industrially in determining the thickness of paper   It is graduated to 0 01 mm   The average of fifteen to twenty measurements is used in calculations

The apparatus used for determining the rate of flow is indicated in Fig 2   The glass tube is a Folin microburette as used in blood sugar determinations, with a capacity of 5 cc , graduated to 0 02 cc but readable to 0 01 cc   The glass stop-cock is cut off and a right angle tube fused on in its place, this tube being of such a length that the distance from the 0 00 graduation to the center of the bend is 100 cm   The details of the apparatus designed to hold the membrane are shown in Fig 3   The diameter of the orifice through which the water passes is 15 75 mm , consequently this becomes the diameter of the membrane disc tested and is a value used in computation   To determine the rate of flow, the membrane to be tested is cut to the appropriate size with a steel punch and placed in the depression in A The rubber washer, also with a hole 15 75 cm in diameter, is placed above it   The flange B is then placed over it with the locking device D fitted together so as to insure against damage to the membrane by rotation of B The collar C is then tightened and the rubber stopper on the burette secured in place   This last operation is done with the holder submerged in water to prevent the formation of air bubbles   The burette is then filled about 1 cm above the zero mark with water and the level is allowed to fall to 0 00   When that point is reached, a stop-cock is started   After an appropriate time the volume of water passed is noted   For membranes between 40 and 200 mμ, this time is usually 10 minutes   Membranes with a pore diameter of less than 10 mμ may require as long as 18 hours before the passage of sufficient water to give an accurate reading   The average pressure producing the flow is determined by measuring the distance from the zero mark on the burette to the point where the reading was made   Since the zero mark represents a pressure of 100 cm , the average pressure be-

comes (100 minus half the distance of fall) A volume/pressure curve can profitably be constructed by which average pressures can be read directly as a linear function of volume For determining the rate of flow through membranes with an average pore diameter of more than 200 mμ, a 50 cc burette is used in the same manner

The water content of the membrane (equivalent to the pore volume) is determined by weighing the same membrane in the wet and dry states Discs 3 15 cm in diameter are blotted with absorbent paper to remove surface water and are rapidly weighed in small, covered, Petri dishes using an analytical balance They are then dried for 48 hours over sulfuric acid in a vacuum desiccator, and reweighed

Since sterile membranes are required for work with viruses and since a slight contraction of the membrane takes place during sterilization, all the measurements given above are made on membranes which have been sterilized by steaming for 1 hour in an autoclave at 0 pressure

Determination of Maximum Pore Size

There is no satisfactory method available by which the size of the largest pores in a membrane can be estimated Elford (4) recommends a method based on the application of Cantor s formula

$$r = \frac{2\sigma}{p}$$

where r is the pore radius, σ the surface tension air/water, and p the pressure required to force air through the wet membrane, as indicated by the escape of air bubbles from a submersed membrane under a measured air pressure In our experience, this method was found applicable only to very permeable membranes and even then readings could be obtained accurate only to a pressure of about 5 pounds per sq in To force air through a water filled capillary 50 mμ in diameter several hundred pounds pressure per square inch is required, which is much in excess of the strength of any membrane To reduce this pressure Bechhold, Schlesinger, and Silbereisen (23) substitute iso butyl alcohol and water mixture for the air water system The system (isobutyl alcohol saturated with water, water saturated with isobutyl alcohol) has a surface tension of 1 85 dynes per sq cm, as
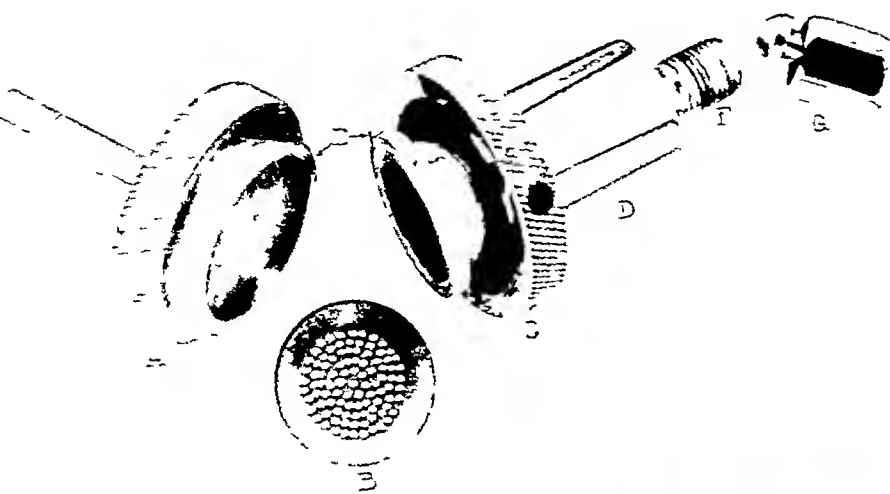
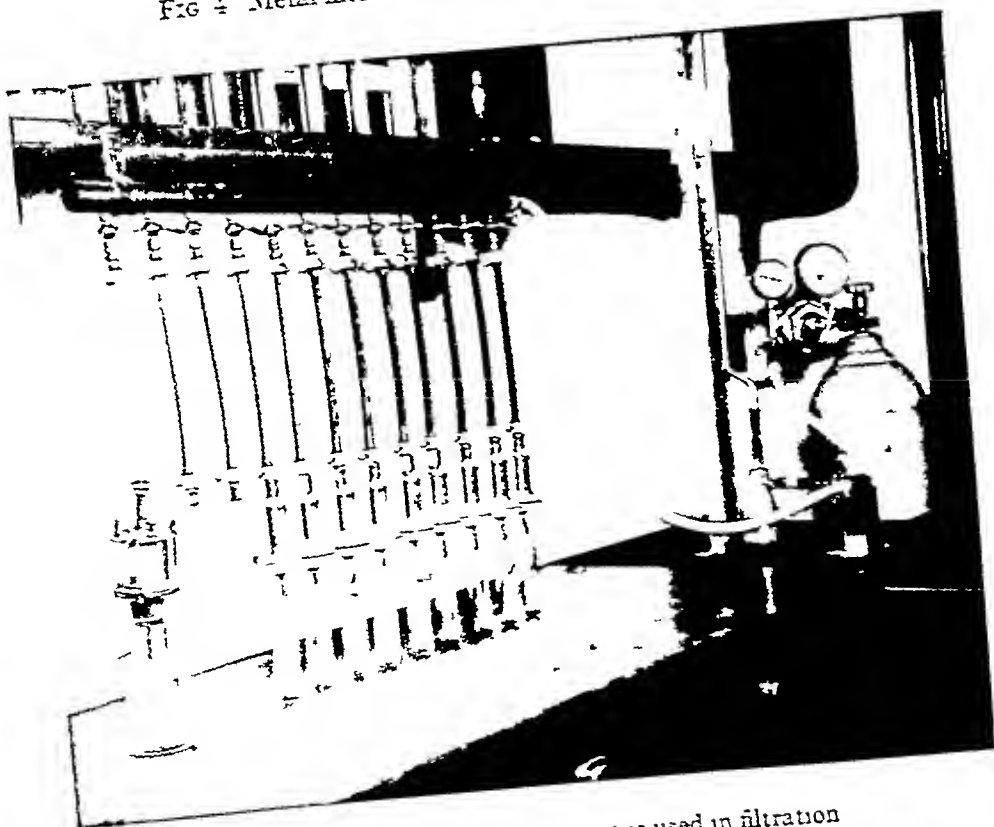Fig 4 Metal filter showing details of different parts

Fig 5 Arrangement of apparatus used in filtration

contrasted with 73 dynes for the air/water system, and consequently correspondingly lower pressures can be used   However, it was found that collodion membranes exposed to isobutyl alcohol became swollen and softened and consequently the method could not be applied

The Use of Membranes in Ultrafiltration

The filters used are similar to those described by Elford (20) and are illustrated in Fig 4   The body of the filter and the collar are made of brass and are chromium plated   The perforated plate, intended to support the membrane, is made of monel metal or stainless steel A rubber washer with a hole 27 mm in diameter is prepared from a special rubber sheeting, 1 5 mm thick, manufactured for use as gaskets in hot water lines   Ordinary laboratory rubber sheeting is unsatis factory, since it does not stand autoclaving   Discs of hard filter paper (Schleicher and Schüll No 575) cut to the same size as the membrane discs (38 mm diameter) are also required   All parts of the filter except the membrane itself are sterilized by autoclaving, the mem brane is sterilized by steaming for 1 hour

In practice the procedure is as follows   The metal plate *B* is placed in the portion of the filter designated as *A*   On this is placed the disc of hard filter paper, which protects the membrane and prevents it from stretching, and also permits filtration throughout the whole area of the membrane   Without the paper, filtration will take place only in the areas directly over the perforations in the plate   The mem brane is placed directly over the paper and on this is placed the rubber washer previously described   The barrel of the filter, *D*, is now put in place, with the projection *E* fitting into a corresponding seat in the stem portion, thus preventing rotation   The collar, *C*, is tightened with a wrench, during which process the stem portion, *A*, is secured in a vise   The material to be filtered is introduced through the stem, *Γ*, which is threaded to take the tapered compression fitting, *G*

Filtrations are carried out under positive pressure, using an arrange ment shown in Fig 5   The manifold has twelve outlets, each provided with a pet cock, a length of high pressure rubber tubing, and the compression coupling shown under *G* in Fig 4   The manifold is connected to a nitrogen tank through a reducing valve, with a mercury manometer between the reducing valve and manifold   For pressures

up to 100 cm of mercury, the manometer is used   When higher pressures are required, the valve connecting the manometer is closed and the pressure read directly from the reducing valve gauge

With this arrangement the material to be filtered can be passed through a series of membranes of varying average pore diameters under uniform conditions of pressure

Properties of Membranes as Shown by Ultrafiltration Experiments

Although there is no satisfactory method by which the sizes of the largest and smallest pores in a membrane can be determined, the sharp, clear-cut end-points observed in our filtration experiments suggest that the pores in any membrane are remarkably uniform in size

Inasmuch as these membranes are intended primarily for the study of filterable viruses, the virus of yellow fever was employed in an investigation of their filtration properties   As shown by Bauer and Mahaffy (24) and by Dinger (25) this virus becomes progressively inactive when suspended in a protein-free medium, such as distilled water or saline solution   It therefore becomes necessary to utilize diluents containing a considerable amount of protein, and consequently having a relatively high viscosity   Filtration of such solutions through collodion membranes affords an excellent opportunity to study adsorption phenomena and other factors involved in the filtration of viruses

The virus-containing material used consisted with a few exceptions of animal tissue ground in a mortar and suspended in a diluent which had been previously tested and found to sustain activity for an adequate length of time   This suspension is centrifuged at high speed for 30 minutes and the supernatant decanted off and passed through a Seitz filter   When passage through membranes with an average pore diameter of less than 75 mμ is required, the Seitz filtrate is not suitable for use, since it contains particles large enough to clog the membrane pores   Consequently it is necessary to pass this filtrate through a collodion membrane with an average pore diameter of about 250 mμ before passing it through the less permeable membranes   It was found that a preliminary passage of hormone broth through the Seitz filter pad greatly reduced the adsorption of virus   Attempts to utilize the sand and paper pulp filter described by Barnard and Elford

(29) for preliminary filtration were unsuccessful, since the filter invariably became clogged after the passage of a few cubic centimeters of virus containing suspensions

As emphasized by Elford (26), the adsorption capacity of the membrane for proteins is greatest near the isoelectric range of the protein and is diminished as the reaction becomes more alkaline Since most viruses are undamaged in pH ranges from 7 0 to 9 0, this range is most useful for filtration The adsorption of protein from the virus containing material can be to a large extent eliminated by passing a small amount of alkaline (pH 8 0) hormone broth through the membrane prior to the filtration That the passage of broth facilitates filtration was first demonstrated by Ward and Tang (27), and its use in ultrafiltration is strongly recommended by Elford (26) We have observed, however, that a preliminary passage of broth through membranes alters the filtration end point A series of tests were made in which broth was incorporated into a diluent for the yellow fever virus together with some protein such as serum As will be shown in another communication, when the preliminary passage broth was omitted the end point for the virus was invariably found to be 55 mμ, a figure in agreement with the results of Findlay and Broom (28) When the virus was suspended in a medium exactly similar, but passed through membranes which had previously been treated with broth, the end point was reduced to 50 mμ

To secure information as to the uniformity of pore size in a given membrane and also the range of particle size of the virus, titrations of the virus content were made with a series of membrane filtrates as indicated below Infected brains were ground up in a mortar, suspended in a suitable diluent, and passed first through a Seitz filter and then through a membrane with an average pore size of 250 mμ Such filtrates are usually infective when 0 02 cc is inoculated intracerebrally into mice in a dilution of 1 100,000 It was found that filtrates passing membranes with average pore diameters of 70, 66, and 60 mμ were infective in a dilution of 1 10,000, while 55 mμ filtrates were infective in a dilution of 1 1000 and a 50 mμ filtrate infective in a dilution of 1 100 Filtrates passing membranes with average pore diameters of 45 and 40 mμ contained no demonstrable virus These results indicate that there is a remarkable uniformity

both in the size of the virus-containing particles and in the pore diameters

It was observed that adsorption of protein by the membranes was greater when the diluent contained 5 to 10 per cent serum than if it contained 25 to 50 per cent ascitic fluid   Three samples of ascitic fluid were tested for protein content and were found to vary from 0 6 to 5 0 per cent   In a majority of the filtration experiments the protein content of the stock filtrate was determined and was compared with that of the membrane filtrates   Where the protein content of the filtrates was greatly reduced the acidity was frequently excessive, due to loss of buffering material   The use of Esbach tubes and Tsuchiya's reagent was found most convenient for these estimations   When virus suspensions were filtered through membranes having an average pore diameter of less than 75 mμ, some loss of protein always resulted even though broth had previously been passed through the membrane   Membranes made from the identical stock collodion solution under identical conditions may differ markedly in their adsorptive capacity   A few were encountered which held back practically all protein and so had to be discarded   We have not yet determined the factors responsible for such variations

Obviously, membranes cannot operate in an uncomplicated sieve-like manner   Even though they are as thin as paper, the ratio of the length of the pore to its diameter is enormous   The virus of yellow fever passes through a membrane which has an average pore diameter of 50 mμ and measures 0 15 mm in thickness   Comparing the pore diameter of 50 mμ to its length of 150,000 mμ, the ratio is 1 to 3000   If we consider the surface and capillary forces which operate in such a long and narrow channel, it becomes apparent that a particle able to transverse this passage must have a diameter considerably smaller than that of the pore diameter   Elford (26) has discussed this matter fully and from the results of quantitative experiments with particles of known size concludes that membranes with average pore diameters ranging from 10 to 100 mμ must have pore diameters of two to three times the diameter of the particle to permit its passage   In membranes having an average pore diameter of less than 10 mμ, or more than 100 mμ, this ratio is decreased

It appears from Elford's observations, as well as our own, that a rela-

tively large group of viruses has a filtration end point between 25 and 100 mµ No observations have as yet been made establishing the pore/particle ratio using proteins of known molecular sizes within these limits, since only one protein substance, hemocyanin of the snail, *Helix pomatia*, (Svedberg and Chirnoaga (30)), with a spherical molecule of suitable size is known   To obtain data applicable to this group of viruses, previous investigators have tested suspensions of metal sols and aniline dyes Since the colloidal behavior of these substances differs radically from that of proteins, we believe that the results so obtained may not be strictly applicable to biological materials

Two proteins having molecular sizes smaller than the size of most of the viruses were tested for filtration end points   Crystalline ovalbumin was prepared by the method of Hopkins and Pinkus (31) and was purified by recrystallization   Oxyhemoglobin was prepared from the blood of sheep and monkeys by washing cells three times in saline, laking them with distilled water, and oxidizing by the passage of air   A mixture for ultrafiltration was made as follows

5 per cent hemoglobin suspension	25 0 cc
Hormone broth, pH 8 0	20 0
4 per cent crystalline ovalbumin	20 0 "
Phosphate buffer, M/15, pH 8 4	20 0 '
Distilled water	10 0 "
1 per cent chinosol solution	5 0 '

Since filtration through membranes with average pore diameters capable of holding back albumin is extremely slow, the chinosol was added as a preservative, being especially suitable for this purpose since it does not coagulate protein   The mixture was passed through a Seitz filter and then through a membrane of 400 mµ and, finally, one of 85 mµ average pore diameter   This stock filtrate was divided into portions and passed through membranes ranging from 9 mµ to 2 3 mµ average pore diameter under a pressure of two atmospheres The filtration period was 18 or 19 hours   The results of the experiment are shown in Tables I and II

The oxyhemoglobins from the sheep and monkey were similar in size, passing through pores of 8 mµ but not passing pores of 7 0 mµ diameter   The albumin in each case passed through pores of 6 2

but not through 4 2 mμ diameter   The albumin was detected by the precipitin reaction, while hemoglobin was recognized by its color

Our results obtained on filtration of ovalbumin are in full agreement with those of Elford (26), who also found the end-point to be 6 mμ

TABLE I

Ultrafiltration Experiment with Oxyhemoglobin from Sheep and Recrystallized Egg Albumin

No of membrane	Average pore diameter	Amount of filtrate collected	Filtration time	Presence in filtrates	
				Oxyhemoglobin	Albumin
	mμ	cc	hrs		
128	9 0	7 0	18	+++	+++
125	8 0	5 5	18	+++	+++
120	7 0	5 0	18	0	+++
143	6 2	6 5	18	0	++
144	4 6	6 5	18	0	0
145	3 7	6 0	18	0	0
146	3 2	6 0	18	0	0
156	2 3	4 0	18	0	0

TABLE II

Ultrafiltration Experiment with Oxyhemoglobin from Monkey and Recrystallized Egg Albumin

No of membrane	Average pore diameter	Amount of filtrate collected	Filtration time	Presence in filtrates	
				Oxyhemoglobin	Albumin
	mμ	cc	hrs		
128	9 0	7 5	19	++++	+++
125	8 0	5 5	19	+++	+++
120	7 0	6 0	19	0	+++
143	6 2	7 5	19	0	++
144	4 6	7 0	19	0	0
145	3 7	6 0	19	0	0
146	3 2	4 5	19	0	0
156	2 3	3 5	19	0	0

The results obtained with oxyhemoglobin, however, differ somewhat Elford used hemoglobin from horse blood, and found the end-point to be 10 mμ Consequently he assigns a pore/particle ratio of 2/1

for hemoglobin and 1 5/1 for ovalbumin   The size of the oxyhemo-
globin molecule has been determined by Northrop and Anson (32),
using diffusion measurements, and by Svedberg (33), using ultra
centrifugal analysis   The results obtained are almost identical,
indicating that the hemoglobin molecule has a dissymmetry ratio
of 1 25 and a diameter of about 5 mμ Svedberg also determined the
molecular size of ovalbumin and found it to be spherical with a diam
eter of 4 34 mμ Using these values in connection with our filtration
results, we find a pore/particle ratio of 1 5/1 for both ovalbumin and
oxyhemoglobin

We have had occasion to carry out filtration experiments with several
viruses which have been studied by Elford, and the filtration end-
points are found to be in close agreement   These results will be
published in later communications

SUMMARY

1 The method described by Elford for the preparation of graded
collodion membranes suitable for ultrafiltration was found to give
excellent results, and his findings are fully confirmed

2 A formula is given for the preparation of collodion from which
satisfactory membranes of graded porosity can be prepared

3 The technique and apparatus used in the preparation, and stand
ardization of membranes are described in detail

4 The technique and apparatus required for ultrafiltration experi-
ments are described, and some drawbacks encountered in the experi
ments are discussed

5 The results of ultrafiltration experiments show that the pores
of the membranes are remarkably uniform in size

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THE EXTINCTION OF REFLEXES IN SPINAL MICE OF DIFFERENT AGES AS AN INDICATOR OF THE DECLINE OF ANAEROBIOSIS

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I

The following experiments were undertaken as part of an investigation of the possibility of the occurrence of partial anaerobiosis in new born and suckling mice Several other methods of approach, such as asphyxiation by illuminating gas or by carbon monoxide (Enzmann and Pincus, unpublished) or by nitrogen (Kohn, unpublished data) have been studied and the results will be presented elsewhere It has been noticed in all these experiments on asphyxiation that young mice exhibit a remarkable resistance to lack of oxygen, which is in agreement with the results of earlier experimental work on similar lines (Bert, 1874, Avery and Johlin, 1932, Irving, 1933, *et al*) It occurred to us to initiate asphyxiation through stopping the blood supply to the spinal cord by transecting it at the mid region of the cervical spinal column, definitely below the level of the respiratory center A similar method has been used to produce anemia in definite regions of the central nervous system (Stenson, 1667, Pike, 1909, and other workers) The result of interrupting the blood supply to the spinal cord is a gradual disappearance of all reflexes and final paralysis

II

The head was severed from the body by transecting the neck with a sharp pair of scissors in the region indicated The method is not ideal as it involves a variable amount of bleeding but is preferable to a blow on the head Stunning and killing the animals by a sharp blow on the head usually leads to severe internal hemorrhages which might interfere with reflex activity after the initial shock furthermore a blow cannot easily be delivered with equal force or always at the same point, and for some reason it leads to a more severe shock than transection of

the spinal cord   The timing was done with a stop-watch   The reflex activity was tested by means of mechanical or graded electrical stimulation

In the description of the phenomena following the transection of the spinal cord at the mid-cervical region we shall make use of some of the terms employed by Riddoch (1917), but we shall define the term "spinal shock" as a temporary stopping of all reflex activity of the spinal cord below the level of the transection immediately after the operation

Four sharply defined periods may be distinguished after the transection, which are characterized by the appearance of the following phenomena

1   Immediately following the operation there is *shock* ("period of flaccidity" of Riddoch), during which the animals are perfectly quiet although various groups of muscles, notably those concerned in flexion of the spine, show strong contraction

2   There follows a period of *hyperirritability with spontaneous mass reflexes*, chiefly vigorous kicking of the hind legs and convulsive twitching of the tail, the forelegs are not always involved

3   The period of *mass reflexes*   The animal is perfectly quiet, all spontaneous motions having disappeared   The nervous system is still hyperirritable and each single stimulus throws the whole body into convulsive activity   This period passes gradually into a period of orderly responses resembling those obtained in the spinal frog, although the reflexes are less purposeful   As the spread of nervous impulses diminishes, the animal gradually enters the last period, that is, failure of reflexes

4   The final period, *failure of reflexes*, is characterized by a gradual decline of the reflex activity   While during the preceding period every stimulus causes results in movements of both fore and hind legs, the spread now is much less   Gradually the area from which reflexes may be obtained diminishes, until only stimulation of the base of the tail and upper part of the hind legs leads to response   The threshold increases enormously, while the vigor of response declines till somatic death supervenes

III

There are remarkable age differences in each of these periods, especially in the last one   The period of shock is very brief in all animals but is definitely shorter in very young mice   The recovery is quicker

in newborn than in adult and more complete, extending to the forelegs
in every case It is noted that the shock is severest in the vicinity of
the lesion The differences in duration of this period with the age
have not been studied in detail because the method of timing with a
stop watch does not permit an exact definition of such small differences
The period of spontaneous mass reflexes lasts longer in young animals
and is more complete The differences in duration of this period
between young and adults are also not large The greatest differences

TABLE I

Duration in seconds of the periods during which reflexes may be obtained from
spinal mice of various ages at room temperature (about 22 C)

Age in days after birth	Mean duration of reflex periods	No of observations
	sec	
0	1270	8
1	922	11
2	731	6
3	650	7
4	610	2
5	476	4
6	408	2
7	338	6
9	207	2
12	148	22
15	68	18
18	47	9
30	39	4
40	33	3
120	34	6

are obtained during the last two periods In newly born mice reflexes
may be obtained by stimulation of the skin for about 1,300 seconds
following transection of the cord, while in adult animals all reflexes
die out in 20 to 40 seconds The duration of the periods during which
responses may be obtained from spinal animals at room temperature
is shown in Table I and in Fig 1 The duration of the periods
during which reflexes are obtainable from spinal animals declines
sharply during the 1st week of life and more slowly during the 2nd
week The adult level is reached at or before the 3rd week after birth

The curve representing the experimental results closely resembles that obtained by Kohn (unpublished data) for asphyxiation of young mice with nitrogen, and by Enzmann and Pincus for asphyxiation with illuminating gas, which supports the view that in the extinction of reflexes in spinal mice we are dealing essentially with the same problem as in asphyxia

In the first series of experiments the temperature of the young animals was not measured It is known that the onset of paralysis in cold blooded animals after clamping the abdominal aorta and depriving the lower spinal cord of oxygen, appears much slower than in

TABLE II

Duration in seconds of the periods during which reflexes may be obtained from spinal mice of various ages at a controlled temperature (37 5°C ± 0 5°C)

Age in days after birth	Mean duration of reflex periods	No of observations
	sec	
0	1040	22
1	880	16
2	679	13
3	596	7
4	585	21
6	473	5
7	322	11
10	176	6
12	145	4
14	78	6
40	42	8

warm blooded animals subjected to the same treatment It is also well known (*cf* Pincus, Sterne, and Enzmann, 1933) that newly born mice are practically poikilothermic and that the apparatus for the regulation of the body temperature develops only gradually

In order to take these facts into account a second set of experiments was arranged Young mice of various ages from birth to 40 days of age were kept in an incubator at 37 5°C ±0 5°C until their body temperature, judged by applying a thermometer to the skin, approximated that of adult mice kept under the same conditions The results are shown in Table II and Fig 1 It appears that the deficiency of the

temperature regulating mechanism in young mice is indeed a factor of importance The curve obtained by strict control of the temperature is during the 1st week of life in every instance below that obtained at room temperature The curve is less steep, the differences in the duration of the reflex periods between newly born and adult mice, however, are significant, and disprove the hypothesis of Barrows (1933) that the difference between newly born and adult mice is due to a difference in body temperature
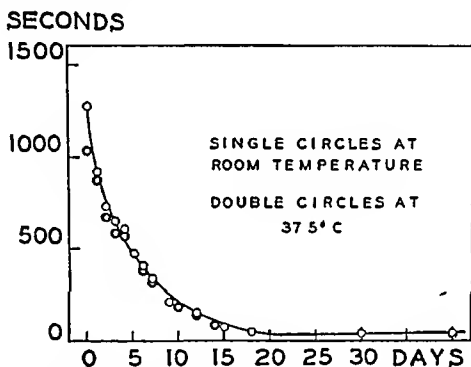

FIG 1 The duration of spinal reflexes (ordinate) in spinal mice of various ages (abscissa)

The assumption that the decrease in resistance of growing organisms to conditions which involve a partial or complete deprivation of oxygen (such as asphyxiation by carbon monoxide, illuminating gas, nitrogen, drowning, spinalization) is due to a decrease in the faculty of partial anaerobiosis with age, seems to offer a logical explanation of our results The literature affords abundant evidence in support of this view

The behavior of spinal animals has been studied extensively (Sherrington 1906 Marshall Hall, 1850, Eckhard, 1881, Owsjannikow 1874 Eckhard, 1881 Pike, 1909, et al) Most of these papers are of little interest in this connection as the

chief emphasis is put on the phenomenon of shock, while we are concerned with the gradual paralysis of the spinal cord after recovery from the shock Sherrington has noted that the activity of the spinal cord is the better controlled the higher the animal's position in phylogeny Babák (1907) found that in larval frogs transection of the spinal cord produces no shock Pike (1909) has reviewed briefly the evidence for the theories of spinal shock and their applicability in phylogeny and ontogeny The chief interest is found in several papers dealing with the stopping of the blood supply to definite regions of the central nervous system, producing asphyxia and consequent paralysis Stenson (1667) clamped the abdominal aorta of a rabbit to interrupt the blood supply to the lumbar region and observed paralysis of the hind legs Further references are given in Pike's paper (1909) Evidence that we are dealing with a decline of partial anaerobiosis in growing organisms is supplied by Wind (1926) and Burrows (1925) who showed that fibroblasts from the heart tissue of a 5 day old chick grew in nitrogen containing only traces of oxygen, while growth was absent in tissue from 10 day old chickens Negelein (1925) showed that in the rat embryo the rate of glycolysis under anaerobic conditions was inversely proportional to age Amerling (1908) found that in *Fugo* and *Rana* the resistance to O_2 lack decreases with age, and Kawajiri (1925) showed that the same conditions hold true for the Japanese salmon *Oncorhyncus mason*

If it is granted that the paralysis of the spinal cord in decapitated mice is due to the failure of blood supply and consequent asphyxia of the nervous elements, it is highly probable that the observed differences in the periods of reflex activity between old and young animals are due to the power of young animals to exist in part under anaerobic conditions and that this faculty declines with increasing age

SUMMARY

In spinal mice the latent period between decapitation and the disappearance of all spinal reflexes decreases regularly with increasing age (Fig 1) Available evidence indicates that this latent period may be proportional to the capacity for anaerobic metabolism at various ages

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THE APPLICATION OF QUANTUM MECHANICS TO CERTAIN CASES OF HOMOGENEOUS CATALYSIS I

By ALLEN E STEARN

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A INTRODUCTION

Recently Eyring and Polanyi (1) have shown how the quantum mechanics can be used to calculate activation energies in good agreement with experimental values   These calculations apply to the large class of reactions which proceed without involving radiation at any step   The energy necessary to break and reform homopolar bonds comes from collisions, and, since the energy found to be necessary is usually not sufficient to break the bonds outright, the bonds must weaken each other as the reacting molecules approach   Thus, upon collision, certain bonds go over into new ones by an adiabatic process

A very general and extremely satisfactory criterion of stability for any configuration of a system of atoms is the potential energy of this particular configuration with respect to that of slightly varied configurations   The lower the potential energy with respect to its values for the other configurations the greater the stability   This is a general thermodynamic criterion and does away with the necessity of trying to picture models of definite kinds of valence binding, though the usefulness and desirability of the latter from a psychological point of view may remain

As an example there is plotted in Fig 1 a graph showing how the potential energy of a system of any two nascent atoms (except those of noble gases) varies with their distance apart   Here it is seen that when the atoms are far apart the energy is constant, *i e* , further separation does not alter it   When they approach sufficiently closely, attractive forces begin to be effective and the potential energy decreases   Upon still further approach the curve passes through a minimum representing the most stable configuration, or the equi

librium distance between the two atoms when the bond is formed
This can be determined in a particular case from X-ray diffraction
data   The "depth" of the "trough" measured from the rim when the
atoms are far apart is a measure of the stability and is related to the
heat of dissociation of the bond   As the atoms approach even more

Kilo cal

Potential energy

20 —

40 —

60 —

80 —

100 —

120 —
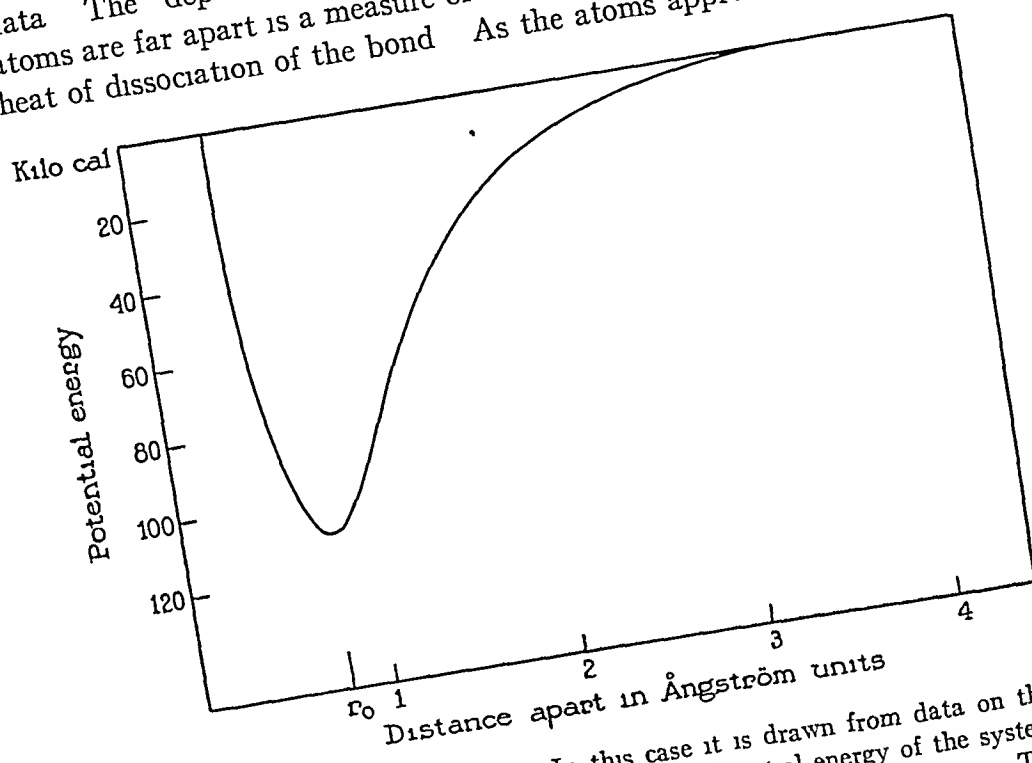
r_0 1

2

3

4

Distance apart in Ångström units

FIG 1 A typical Morse curve   In this case it is drawn from data on the
hydrogen molecule and represents the change in potential energy of the system
(ordinates) as a function of the distance between the hydrogen atoms   The
point of lowest energy (or greatest stability) comes at the equilibrium distance
between the atoms in the ordinary molecule, and the depth of this trough is closely
related to the heat of dissociation of the H—H bond, $i\,e$, the energy necessary
to separate from a distance apart of r_0 to an infinite distance apart   Analogous
curves quantitatively describing any bond of covalent nature between two atoms
may be drawn from the proper data

closely they begin to repel each other strongly, and the potential
energy rises very sharply   Curves of this kind which quantitatively
describe bonds between any pair of different atoms may be constructed
from known data by a simple function proposed by Morse (2) and are
called Morse curves

If, instead of two nascent atoms, two molecules approach and undergo reaction the picture is different   Consider the reaction
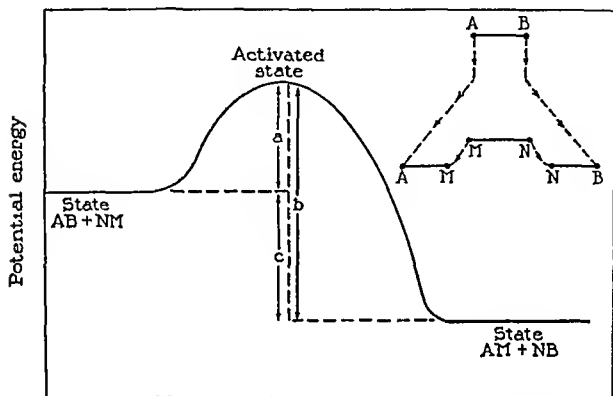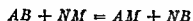
$$AB + NM = AM + NB$$

FIG 2 Showing the change in potential energy (ordinates) of a system of two molecules AB and NM, as reaction to form AM and NB takes place   Abscissae may be thought of as giving the course of reaction AB and NM must approach to within a certain distance before any change in energy takes place (left horizontal portion of curve)   During reaction the energy rises and then falls to a constant value for the new molecular system AM and NB The height a measures the activation energy for the reaction AB plus NM equals AM plus NB while the height b measures this same quantity for the reaction in the reverse direction   The height c is a measure of the heat of reaction   It is exothermic in one direction (as written above) and endothermic to the same extent in the other

The physical process is pictured in the upper right hand corner

If we plot the total potential energy of the various configurations of the four atoms, A, B, M, and N, as this reaction proceeds, we obtain the curve given in Fig 2   Following the course of this curve it will be noted that as molecules AB and NM approach from sufficiently large distances apart there is at first no change in total potential energy

and the curve is flat   Upon closer approach there is a *rise* in potential
energy which increases as reaction proceeds until a maximum value
is reached   The rise in the curve may be pictured as due to the fact
that the incipient breaking of the original bonds against their *full*
bond strength is pitted against the incipient formation of new bonds
which do not reach their full bond strength until completely formed
The height of the "hill" above the original plain is a measure of the
heat of activation and the state of the system at the summit is the
activated state   It is a state of "indecision "   If the original mole-
cules possess enough energy to carry them over the hill the reaction
goes on to completion, if they possess insufficient energy the reaction
does not go and they merely collide without reaction

In passing, one or two facts may be pointed out   (*a*) In general
the heat of activation will not be the same for the reverse reaction as
for the original one   The difference, *i e* the difference in height of the
two flat portions of the curve, is a measure of the heat of reaction
(*b*) The heat of *activation* is the principal factor in determining whether
a reaction will proceed spontaneously or not   (*c*) The heat of *reaction*
is more directly concerned with how nearly a statistical system of
reacting molecules will undergo complete reaction before equilibrium
is reached   Thus if the heat of *reaction* is zero the heat of activation
is the same in either direction and, in the particular example cited
equilibrium would be reached, starting with chemically equivalent
amounts of reagents, when the reaction had proceeded half way to
completion   Such considerations are for statistical systems only
In the individual event the reaction either does or does not go to
completion

Naturally the most satisfactory method of determining any magni-
tude in which we are interested is by direct experimental measurement
As a second choice we measure some related magnitude whose connec-
tion with the one desired involves a minimum of inference   With
thermal measurements one runs into many difficulties   It is true
that the heats of most reactions might conceivably be directly meas-
ured   But these values involve only the system before any reaction
has started and after it has gone entirely to completion and give us no
information about the *reacting* system   In very simple cases activa-
tion energies can be measured   Experimental determinations here

usually involve reaction velocities and thus presuppose a knowledge of reaction mechanism, since, if a reaction takes place in steps it is the velocity of the slowest step which is the determining factor   Even when these values can be given a definite significance, however, they tell us only the maximum height of the "potential hill" (Fig 2) and say nothing about the course of the reaction nor the configuration of the system in the activated state   Furthermore, since they involve measurements at different temperatures one runs into the possibility that the limiting, or slow step at one temperature may not be the same as that at the other temperature

By the method of Eyring and Polanyi one calculates the potential energy of any specified configuration of atoms   Even in the present unperfected state the information obtainable in this manner is of high value   Thus, for a particular postulated mechanism of reaction, the complete course from start to finish can be followed, by a sufficiently exhaustive study the actual mechanism can be ascertained (from the possible mechanisms one eliminates all except one, i e that of lowest activation energy), the calculations are based on very fundamental experimental data of a general nature

The quantum mechanics has uncovered a hitherto unsuspected source of binding energy—a resonance of electron pairs between atoms due to a rapid interchange of electrons among themselves   This gives a more definite picture of the electron pair bond   The energy of any configuration is, then, given in terms of two contributions—an electro static force, always attractive and usually termed the coulombic part, and a resonance force, which may be either attractive or repulsive, depending on whether the electrons are paired or not, usually called the interchange contribution   The pairing of electrons may be roughly thought of as due to the fact that they are all spinning and are thus small magnets   The binding, of course, does not arise from this magnetic effect but is an electrical attraction   Only two electrons of opposite spins pair, and any other electron is then repelled   In the ordinary bond it is found that about 14 per cent of the bond strength is due to the coulombic contribution and about 86 per cent to the inter change   The particular distribution between these two factors makes no difference on any initial or final state of the system but is of signifi cance in the activated state as the value of the activation energy is appreciably affected by this distribution

By this method calculations are fairly easily made for systems containing four reacting atoms, and they can be quite practically made for those containing five or six atoms   For larger molecules the *complete* problem is, in practice, almost impossible of solution   Even here, however, much valuable information can be obtained by confining oneself to the *reacting groups*

When we make this simplification the most important factor we are neglecting is the steric effect of the non-reacting parts of the molecule   Such effects are of much more importance in some cases than in others   They are particularly important for certain classes of organic reactions [1]

B Catalysis

Specific reaction velocities can obviously be increased in two ways   The number of molecules possessing sufficient energy to surmount the barrier, or potential hill, may be increased   The temperature effect on reaction velocity comes through this mechanism, and, as is well known, data from temperature coefficients of reaction velocities are the basis of the ordinary method of measuring activation energies   There may, however, be varying environmental conditions which alter the height of the barrier   These are catalyzing conditions, the catalyst being negative if the height be increased and positive if it be decreased

The statement was made above that the distribution of energy between coulombic and resonance was quite significant in the activated state, *i e* when the system is just at the top of the hill   Large polarizabilities resulting in appreciable permanent dipoles, as well as extra charge due to ionized condition of reacting groups, play a rôle in increasing the per cent of the total energy which is coulombic, and therefore in changing the height of the hill   This energy can be estimated as

$$- \Sigma \frac{e_i e_j}{r_{ij}}$$

i e , the product of each pair of charges over their separation in a

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[1] In certain cases even these effects can be treated by quantum mechanics and the author has some specific cases under investigation

dielectric of unit constant   The sum is of course algebraic and is a re
pulsion for like charges and an attraction for opposite   In any other
dielectric medium the constant must be considered, which, for water
at the distances involved, approaches the optical value of 3 in place
of the static value of 80, which is the constant for larger distances
of separation (3)

C Hydrolysis of the Carbon Nitrogen Linkage

The reaction

$$\equiv C - N = + HOH \rightarrow \equiv COH + = NH$$
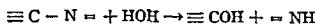
was investigated   Thus we are in reality investigating the hydrolytic
splitting of a C—N bond and neglecting steric effects of the other groups
attached to the carbon and nitrogen   If to the former there is
attached a carbonyl oxygen and to the latter one hydrogen we have
essentially the peptid linkage   Were we to be interested in the
absolute values of the activation energies in this paper such steric
effects should not be neglected, though they are by no means easy to
estimate [2]   In this paper, however, we shall be interested in catalytic
effects, i e in lowerings of activation energies rather than in their
absolute magnitudes, and the results, it is felt, can be thought of as
applicable to the peptid linkage even though the nature of the par
ticular substituents attached to the C and the N are not specified

The reaction may be diagrammatically indicated as follows

(a)	(b)	(c)
Reaction not yet started	Some intermediate state one of which is the acti vated state	Reaction completed

As the O—H group approaches the C—N group these two linkages tend
to weaken and other linkages begin to form namely C—O, C—H,

[2] Even in such cases the order of magnitude can be obtained   Thus from some
unpublished calculations it is found that substitution of a C—O for a C—H bond
on one of the carbons of a C—C bond lowers the activation energy necessary to
break the C—C bond by about 3 kilo cal

N—O, and N—H   These are only incipient tendencies and furnish only a small amount of energy whereas the breaking of the original O—H and C—N bonds must be accomplished against their full strength   Thus the potential energy of the system will rise with the approach of the reacting groups so that they will be repelled unless they have sufficient kinetic energy to balance this repulsion   The minimum energy required is the energy of activation   It is the energy just necessary to carry the reacting system over the potential hill (Fig 2)

Now the binding energy, E, of a configuration of four atoms involving one valence each is given by the expression

$$E = A_1 + A_2 + B_1 + B_2 + C_1 + C_2 + \tag{1}$$
$$\overline{\sqrt{\tfrac{1}{2}[(\alpha_1 + \alpha_2 - \beta_1 - \beta_2)^2 + (\alpha_1 + \alpha_2 - \gamma_1 - \gamma_2)^2 + (\beta_1 + \beta_2 - \gamma_1 - \gamma_2)^2]}}$$

Where $A_1 + \alpha_1$ (see Fig 3) is the total binding energy of one of the atom pairs and is a function only of the distance apart of this pair   This is directly read from the corresponding Morse curve for the particular atom pair   The same thing is true for $B_1 + \beta_1$, $B_2 + \beta_2$, $C_1 + \gamma_1$, etc   Here the A's, B's, and C's give the coulombic energies and the α's, β's, and γ's give the interchange energies   The distribution of energy in ordinary cases has been found to be 14 per cent coulombic to 86 per cent interchange   Thus it is a comparatively simple matter to get the energy of any configuration   To obtain the activation energy one calculates the energies for a number of configurations as the O—H and the C—N groups approach, varying all the factors including angle of approach   One can then construct a potential surface or series of surfaces and the lowest potential hill over which it is *necessary* for the reacting system to go will measure the activation energy

The equation proposed by Morse to give the potential energy, E, of a pair of atoms as a function of their distance apart, r, (see Fig 1) has the form

$$E = D' (2e^{-a(r-r_0)} - e^{-2a(r-r_0)})$$

Here D' is the heat of dissociation of the bond, D, plus the half quantum of vibrational energy it has in its lowest state, $i\,e\,, \tfrac{1}{2}\,h\,w_0$, where w_0 is the vibration frequency in the lowest level r_0 is the equilibrium

value of r in the fully formed bond, and a is given by $a = 0\,1227$ $w_o (M/D')^{\frac{1}{2}}$, M, the reduced mass being defined by

$$M = \frac{M_1 M_2}{M_1 + M_2}$$

M_1 and M_2 being the atomic weights of the two elements forming the bond

Table I gives the constants used in obtaining the Morse curves for the various bonds involved in the reaction here studied D and D' are in kilogram calories, r_o is in Ångstrom units, and w_o in wave numbers

For this reaction the activation energy comes out about 31 9 kilo cal   The potential energy change during the course of reaction is

TABLE I

Constants Used in Morse Equation

Bond	D	D	r	a	w
C—N	66 4	67 87	1 47	2 090	1035
C—H	92 3	96 46	1 12	1 874	2930
C—O	81 8	83 27	1 43	1 940	1034
N—H	96 9	101 55	1 06	2 050	3270
N—O	38 5	40 2	1 36	3 357	1191
O—H	113 1	118 3	0 97	2 135	3660

given in Fig 4, Curve A   The ordinates are potential energy values of the reacting system while abscissae cannot be so easily defined They really represent the succession of configurations of the system during the course of reaction for which the potential energy is a minimum   The curve consists of two horizontal parts and a center hill   The abscissae along the left hand horizontal part may be thought of as giving the approach of the two original groups, and, those along the right hand horizontal part as giving the separation of the products formed in the reaction   The abscissae in the center give configurations during reaction   Complete graphical representation would require a four dimensional figure and the points on the curve in Fig 2 are taken from such a figure (or series of three dimensional ones) and selected to give the lowest potential hill over which the reaction must go

In passing it may be pointed out that the center of the hill seems in reality to be the floor of a shallow basin whose rim is somewhat over 3 kilo cal above the floor   This might mean a hydration with corresponding heat of dissociation   The interpretation in any case is suggestive   Thus dissociation of this rather unstable complex in one direction would yield water and a peptid while in the other direction it would yield a substituted amine and an alcohol or carboxyl   In the present case this basin is of little importance as its depth is small compared with the height of the hill which must be surmounted before reaching it   However, were the hill itself significantly lowered the same basin would attain much significance and might represent the type of bond which obtains, for example, in the reaction between proteins and dyes   The factors which alter the height of this hill will be treated in the next section   But further consideration of the basin will not be included in the present paper

D Catalytic Effect of Hydroxide and Hydrogen Ions on Reaction

The mechanism of the catalytic effect of these ions can be ascribed to a redistribution of charge either entirely or in part by induced polarization, with a consequent increase in coulombic attraction and concomitant lowering of total potential energy   Qualitatively it may be seen at once that the hydroxide ion may be expected to have the greater effect for here we can definitely locate one electronic charge on the oxygen of the reacting O—H group whereas the hydrogen ion produces its effect by induced polarization [3]

The calculations under these conditions are by no means as straight forward as for the first case, the results of which have been given above   It is thus worth while to indicate their nature   In the calculations referred to in Section C, the distribution of coulombic and

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[2] It may be questioned why, if we view the hydrogen ion as essentially OH_3^+, we might not have the charged hydrogen as an essential constituent of the reacting O—H group with, at first sight, even greater catalytic effect than the hydroxide ion   A writing out of the reacting configuration, however, would show that as the reaction goes to completion one of the other O—H bonds in the OH_3^+ would also have to be broken and for a hydrogen more remote from the reacting one, which is very improbable   The actual effect of this ion may better be looked upon as an induced coulombic effect

interchange energy was taken as 14 per cent and 86 per cent respectively of the total energy This has been found experimentally to hold in many of the cases studied For a bond between two hydrogen atoms where induced polarization is small Sugiura (4) finds a corresponding distribution of 10 per cent to 90 per cent and this changes with polarizability being 22 per cent for the Li—Li bond (5) and 28 3 per cent for the Na—Na bond (6) We shall therefore take 10 per cent and 90 per cent as a starting value and correct for additional coulombic energy The assignment of charges can be approximated by a knowledge of the dipole moments of the bonds From the values for these moments (7) the following charges were assigned to the atoms of the reacting groups

$$C + 1 2 \times 10^{-10} \text{ e s u}$$
$$O - 0 6 \times 10^{-10} \text{ `` ``}$$
$$N - 1 0 \times 10^{-10} \text{ `` ``}$$
$$H + 0 33 \times 10^{-10}$$

For the activated configuration (Fig 3) the coulombic energy in kilogram calories per mol is

$$\left(\Sigma - \frac{e_i \, e_j}{r_j} \right) \frac{14 \, 35}{3}$$

if e is given in e s u and r is in Ångström units Here 3 is the optical dielectric constant of the medium This added coulombic energy in the case of the activated configuration amounts to 5 52 kilo cal This would bring the coulombic per cent from 10 per cent to 13 6 per cent giving an activation energy of 32 7 kilo cal in place of the 31 9 kilo cal obtained by using the 14 per cent to 86 per cent distribution Thus it is thought that one can in this way get a reasonable order of magnitude for catalytic effects [4]

[4] To verify this it is merely necessary to take the distances given in Fig 3 and read off the various bond energies from corresponding Morse curves If R_1 R_2, R_6 are these readings and if λ and Y are the respective fractions giving coulombic and interchange energy then Equation 1 may be written

$$E = \lambda \, [R_1 + R_2 + \quad + R_6]$$
$$+ Y \sqrt{\tfrac{1}{2}[(R_1 + R_2 - R_3 - R_4)^2 + (R_1 + R_2 - R_1 - R_3)^2 + (R_3 + R_4 - R_1 - {}_6)^2]}$$

In this form the adjustment of λ and Y and E for induced coulombic energy becomes quite simple

Table II gives basic data from which the extra coulombic energy
the polarized systems, $\imath$ c , in presence of H^+ or OH^-, are calculated
Values of α are obtained by assigning the same refraction to every
ectron in the valence shell of an atom and assuming all unshared
ectrons to be polarized with the bond   Thus the C–O   bond
raction $= \frac{1}{4}$ C $+$ 5/6 O where C and O are atomic refractions of
rbon and oxygen respectively   For alkaline solutions, where the
arged oxygen would not polarize its own bond with appreciable
crease in energy, the polarized bond would be N—H in configuration
ind C—N in configuration 10, for examples   The configurations are
mbered arbitrarily as plotted as abscissae in Fig 4 and one of them,

TABLE II

Bond	Atomic distances in Ångström units						Bond polariz ability $\alpha \times 10^{24}$
	Equilibrium	Configuration No					
		3	4	7	10		
C—O	1 43	1 50	1 50	1 60	1 70		0 89
C—N	1 47	1 64	1 63	1 45	1 41		0 77
N—H	1 06	1 06	1 12	1 18	1 18		0 94
O—H	0 97	1 59	1 40	1 10	0 97		1 05
C—H	1 12	2 03	1 90	1 74	1 68		0 65
N—O	1 36	2 07	2 07	1 97	1 96		1 17

nfiguration 4, is shown in Fig 3   They are merely different suc-
ssive configurations of minimum potential energy assumed by the
stem as the reaction takes place   In the case of acid solutions,
ere the proton itself is not part of the reacting system, two bonds
ll be polarized for every configuration, e g , C—O and N—H in
nfiguration 3 C—N and O—H in configuration 10, etc
The induced atomic charges, c_a, are calculated from the relations

$$\mu = \alpha F = c_a r$$

$\,=$ induced dipole moment) whence

$$c_a = \frac{\alpha F}{r}$$

α = polarizability, F = field intensity in e s u , and r = distance in centimeters

But

$$F = \frac{e_p}{3r^2}$$

e_p = polarizing charge in e s u , and 3 = dielectric constant in water at distances involved in the configurations studied   Tables
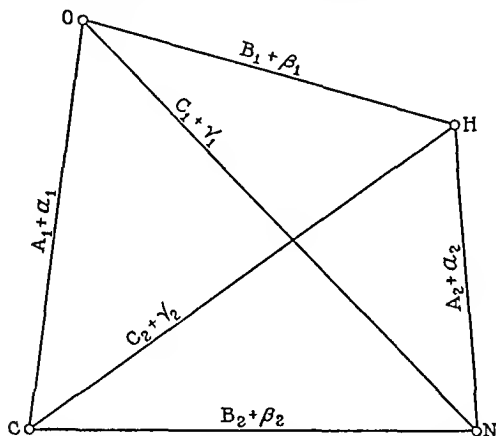

FIG 3 One of the configurations of the system, drawn to scale, for which the energy was calculated This in fact was the configuration of highest energy or the activated configuration, for the case under consideration

may be made for various values of r and thus of F giving the induced and total charges for any configuration For hydroxide ion a conservative estimate, for all configurations studied, of the value of r is about 2 Å u (see bottom row of Table II) This approaches very near to the actual conditions, and Table III contains the results of calculations for certain of the configurations on such a basis

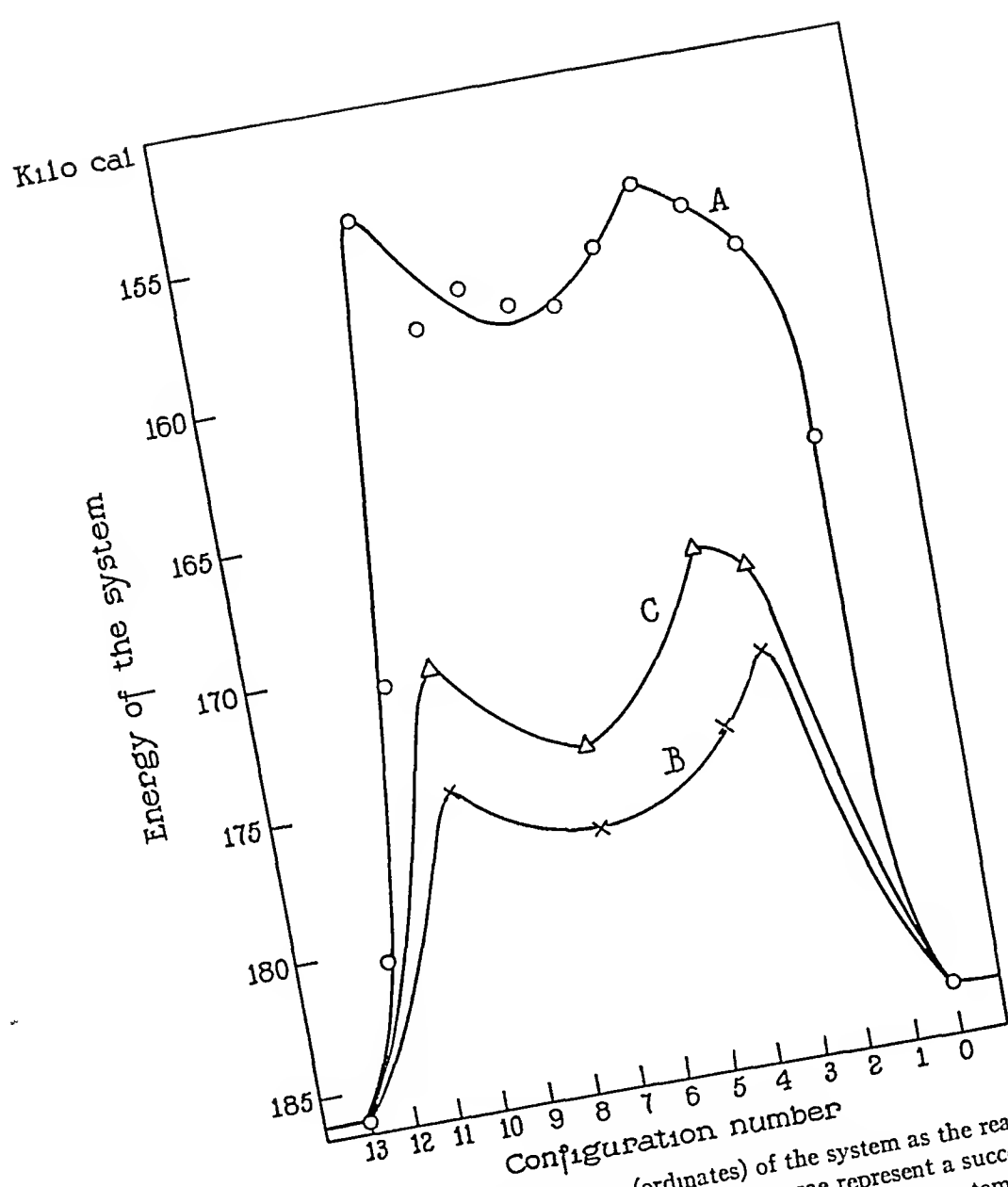These energy values (the last column of Table III) are plotted as

FIG 4 Showing the potential energy (ordinates) of the system as the reaction C—N + O—H = C—O + N—H takes place   Abscissae represent a succession of configurations of minimum potential energy through which the system must pass in the course of the reaction   Their numbering is arbitrary and happens to correspond to that according to which the reaction was originally worked out In this figure, configuration 13 represents the state of the system before reaction has begun, and configuration zero the state after the reaction has been completed Curve A is for neutral water in absence of OH⁻ or H⁺ ions (an ideal case), Curve B is for reaction in alkaline solution, and Curve C for reaction in acid solution In Case A, configurations 4 and 10 are nearly indistinguishable and the activation energy could be obtained from either   In Case B configuration 3 is the activated state, while in Case C configuration 4 is the activated state   As shown here the reaction is almost isothermic, being endothermic to the extent of about 1 4 kilo cal.

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Curve B in Fig 4 The heat of activation is $186\,2 - 171\,7 = 14\,5$ kilo cal This value should not be compared directly with the value, ca 32, for neutral water since the OH ion concentration will always be much lower than the effective water concentration in dilute aqueous solutions Should we arbitrarily take the latter value as 55, then

TABLE III

Hydroxide Ion Catalysis

$F = 4 \times 10^5$ e s u corresponding to polarizing distance of 2 Å u

Configuration		Charge $\times 10^{10}$ e s u.				Polarized Bond	Polarization coulombic energy	Total energy of configuration
		C	N	O	H			
							k_f cal	k_f cal
Before reaction		—	—	—	—	—	—	−186 2
3	Induced	—	0 34	—	0 34	N—H	−20 0	−171 7
	Total*	1 2	1 34	4 77	0 67			
4	Induced	—	0 34	—	0 34	N—H	−20 9	−174 1
	Total	1 2	1 34	4 77	0 67			
7	Induced	0 22	0 22	—	—	C—N	−18 4	−177 0
	Total	1 42	1 22	4 77	0 33			
10	Induced	0 14	0 14	—	—	C—N	−18 8	−174 8
	Total	1 34	1 14	4 77	0 33			
After complete reaction		—	—	—	—	—	—	−184 8

* This total charge is obtained by adding the induced charge to the fundamental dipole charges given above The essential correctness of these values is rendered highly probable since they are in agreement *independently* with the dipole moments involved and the basic 14–86 distribution of coulombic to interchange energy in the neutral system

Table IV would give the apparent relative activation energies at various pH values

With acid catalysis we proceed in the same manner While the problem is slightly more complicated than in the case of alkaline catalysis much of the uncertainty is eliminated by the fact that there

will be no interchange repulsion in the case of the proton   For configurations 3 and 4 it will form part of a substituted ammonium ion while for configuration 10 it will form part of an oxonium ion   Only

TABLE IV

pH	Apparent activation energy
	kg cal
(Non-ionized water)	31 9
10	22 8
11	21 3
12	20 0
13	18 5
14	17 0

TABLE V

Acid Catalysis

Configuration		Charge $\times 10^{10}$ e s u				Polarized bonds	F	Polarization coulombic energy	Total energy of configuration
		C	N	O	H				
							e s u	*kg cal*	*kg cal*
3	Induced	0 18	0 89	0 18	0 89	{ C—O	3×10^5	−16 9	−168 5
	Total	1 38	1 89	0 78	1 22	{ N—H	10^6		
4	Induced	0 18	0 84	0 18	0 84	{ C—O	3×10^5	−15 8	−167 6
	Total	1 38	1 84	0 78	1 17	{ N—H	10^6		
7									−174 0*
10	Induced	0 17	0 17	1 08	1 08	{ C—N	3×10^5	−15 8	−170 3
	Total	1 37	1 17	1 68	1 41	{ O—H	10^6		

* In this configuration neither N—H nor O—H are near their equilibrium bond distances apart (see Table II) so one cannot definitely place the proton either on the N or on the O   Arbitrary calculation on the one assumption leads to a value − 173 kilo cal for the total energy of the configuration while − 175 kilo cal is obtained on the other assumption   This is of minor importance since configuration 4 is the activated state here

in such a configuration as 7 will there be much doubt, and so far as activation energy is concerned this is of less importance than 4 or 10 (the former being the activated configuration under these conditions)

Thus from the structure of the ammonium ion and oxonium ion we can fairly definitely locate the polarizing charge Table V gives results for acid catalysis analogous to those of Table IV for alkaline catalysis We now have two bonds polarized and with different values of field intensity, as the proton will belong either to the ammonium or the oxonium system and will not be symmetrically placed

The heat of activation, then, is 186 2 — 167 6 or 18 6 kilo cal These results are plotted as Curve C, Fig 4

E Relative Catalytic Effects of Hydroxide and Hydrogen Ions

The activation energies of hydroxide and hydrogen ions are, then, respectively 14 5 and 18 6 kilo cal , a difference of 4 1 kilo cal If pH were in both cases a measure of the effective concentrations this would correspond, at 40°C , to relative speeds of hydrolysis of

$$e^{\frac{4100}{626}} = 700$$

However, while pH is such a measure for hydroxide ion as it enters as one of the reagents, it is not so for hydrogen ion whose effective concentration is much greater than the value given by pH This statement must not be interpreted as meaning that catalytic effect in acid solutions is independent of pH Rather the difference in the two ionic effects can be pictured as follows The maximum effect of OH ion comes when it enters as one of the reagents rather than plays a purely inductive rôle As such the pH would measure its effective catalytic concentration The hydrogen ion, on the other hand, cata lyzes by purely induced charges on the reacting atoms pH measures its activity throughout the solution, but there may be a tendency for it to concentrate selectively at the reacting points in the molecules If by hydrogen ion one means strictly oxonium ion there will be no such tendency, but if one means, more loosely, proton, then the forma tion of a basic nitrogen as the hydrolysis proceeds would induce proton concentration in the sense of the proton transferring from oxonium ion to a sort of substituted ammonium ion If, for a particular con figuration such as the activated one, the distribution ratio of proton between water and nitrogen of the substituted ammonia remains sensibly constant, then, even though the catalytically effective con

centration of proton may differ from that given by pH measurement, nevertheless the change in reaction velocity with hydrogen ion concentration will be proportional to the change of the latter as measured by pH   Thus to get at this effective H ion concentration, even though in a rough way, one must investigate the distribution of proton between water and a properly substituted ammonia

In the activated state, configuration 4, we have practically an acetamide nitrogen   Thus we have

$$RCONH_2 + OH_3^+ \rightleftharpoons RCONH_3^+ + OH_2$$

$$\frac{(RCONH_3^+)\ (OH_2)}{(RCONH_2)\ (OH_3^+)} = K$$

But

$$(OH_3^+) = \frac{K_w}{(OH^-)}$$

or
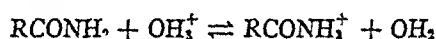
$$\frac{(RCONH_3^+)(OH^-)}{(RCONH_2)} = \frac{K\ K_w}{(OH_2)} = K_b \text{ and } K = \frac{K_b(OH_2)}{K_w}$$

where K_b is the basic ionization constant of the amide

Now at 40 2°C   (8) K_b for acetamide is $3\ 3 \times 10^{-14}$, for acetanilide it is $4\ 4 \times 10^{-14}$, and for urea $3\ 7 \times 10^{-14}$ K_w is $ca\ 3 \times 10^{-14}$ Thus, approximately,

$$K = (OH_2) = 55$$

and the effective concentration of hydrogen ion is not far from 55 times its actual concentration   This would cut down the speed ratio of OH^- to H^+ catalyzed hydrolysis from 700 to around $\frac{700}{55}$ or 13

One might expect therefore this speed ratio to lie between 13 and 700 and much nearer to the former value at 40°C [5]

---

[5] It may well be suggested that this argument about the proton distribution would not apply to such a configuration as 10 where the O—H separation has its equilibrium value of 0 97 Å u and N—H has a separation too great to give it the consideration of an amide configuration   In this case where no doubt the effective and actual H^+ concentration must be considered the same the energy of the system is lower, $-170\ 3$ kilo cal in place of $-167\ 6$ for configuration 4

It is very suggestive that the results of Northrop (9) on the hydroly-sis of gelatin at 40° show that, for sufficiently high hydrogen or hydroxide ion concentrations, the velocity ratio, which has been calculated for splitting the peptid linkage as between 13 and 700 and probably much nearer the former value, seems to be about 30

In nearly neutral solutions the hydrolysis proceeded some 300 to 400 times as rapidly as predicted by linear dependence on H^+ or OH^- concentration This was attributed to a difference in specific reaction rate between the ions of gelatin and the "uncombined" material though no reason for this was suggested at the time A ratio of 300 at 40° corresponds to an activation energy difference of about 3 6 kilo cal, an amount easily possible and perhaps due to a combination of two factors The shortening of protein "spirals" in the isoelectric condition (10) may involve both steric effects in interchange repulsions and valence angle changes Both involve energy The order of magnitude may be seen by the facts that (1) substitution of a C—O bond for a C—H in ethane decreases the activation energy required for hydrolytic breaking of the C—C bond by over 3 kilo cal (11), the actual rotation of a methyl group about the C—C bond axis in methane through 60° from the stable configuration involves about 0 34 kilo cal (12), a distortion of 5° in the angle of directed valence in a water molecule involves about 8 kilo cal (12), etc Thus, during the pH range through which gelatin is passing from the anion state through the isoelectric state and into the cation state there may well he a goodly portion of this range where the reaction velocity for hydrolysis is nearly independent of pH

The results of Wijs (13) fit into this picture very suggestively The bonds involved are not identical, but the values of r_o are strikingly nearly the same so that the activated configurations will differ only very slightly This is shown in Table VI Now in such a case we might well expect the relative effects of OH^- to H^+ to be very nearly

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The 55 fold increase mentioned above would correspond to nn npparent energy change in the system of 2 5 kilo cal bringing configuration 4 to an npparent value of − 170 2 so that it does not drop under configuration 10 In other words passing through configuration 10 even though we cannot speak of higher effective H^+ concentration will not 'hold up" the reaction with respect to configuration 4

the same *except* that we have now no amide group to alter the effective H^+ ion concentration Wijs found, at about $25°C$, that the OH^- ion was some 1370 times as effective as the H^+ ion This corresponds to a difference in activation energies of 4 3 kilo cal as compared with 4 1 kilo cal calculated here This does not, of course, mean that the activation energies of the two hydrolyses are the same but that the calculation of the relative catalytic effects of H^+ and OH^- should be the same were it not for the amide N in the activated state of the peptid hydrolysis

In a second paper the possible prosthetic action of certain groups will be considered from the point of view of their dipole effect on the coulombic energy of an activated configuration

TABLE VI

Bonds involved in peptid hydrolysis		Bonds involved in hydrolysis of methyl acetate	
Bond	r_o	Bond	r_o
	Å u		Å u
C—O	1 43	C—O	1 43
C—N	1 47	C—O	1 43
O—H	0 97	O—H	0 97
N—H	1 06	O—H	0 97
C—H	1 12	C—H	1 12
N—O	1 36	O—O	1 32

SUMMARY

1 The activation energy for the hydrolytic breaking of a peptid linkage has been calculated from quantum mechanical considerations

2 The relative catalytic effects of H^+ and OH^- and the mechanism of these effects have been calculated and discussed

3 These effects are both qualitatively and semiquantitatively in agreement with experiment

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TEMPERATURE CHARACTERISTICS FOR THE METABOLISM OF CHLORELLA

II THE RATE OF RESPIRATION OF CULTURES OF CHLORELLA PYRENOIDOSA AS A FUNCTION OF TIME AND OF TEMPERATURE

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I

Emerson (1926–27) has demonstrated the presence of two types of respiration in *Chlorella pyrenoidosa* and other green algae   The first is the normal respiration of the plant during the course of which a photosynthetic product is consumed, and is exhibited when a culture is suspended in Knop's solution   Iron-binding inhibitors such as cyanide slightly increase its rate   The second type of respiration is apparent on the addition of glucose to the suspending medium, when the respiration of the organism is doubled   The mechanism responsible for this increase differs from that concerned with the normal respiration, however, in that it may be inhibited by the action of cyanide

In a previous paper (Crozier, Tang, and French, 1934–35) dealing with *Chlorella*, the temperature relations were determined for respiration in Knop's solution to which glucose had been added   We now consider the "normal" respiration of the organism, in order to determine the time course of the reaction at constant temperature, the respiratory quotient, and the temperature characteristic   We shall discuss these topics in the order mentioned   It is to be noticed that the conditions of these observations are such that constant rates of respiration are not to be expected   The cells have been actively and continuously engaged in photosynthesis, but with the beginning of the experiment they are transferred to darkness, metabolism then involves a declining store of photosynthetic reserves

II

Two series of experiments were made without added glucose, one on June 1–3 (C S F –P S T), and one on Oct 13–16 (C S F –H I K), 1933   The technique was essentially the same in both series, differences of detail being given in Table I

The cells were from the same strain of *Chlorella pyrenoidosa* previously used, which were given us by Mr William Arnold   They were grown in Knop's solution as previously described, 30 cm from three

TABLE I

Data for Experiments of Series I and II on Respiration of Chlorella

Series	I	II
Date of experiment	June 1	Oct 13
Temperature of growth	22°C	20°C
Age of cells	5–6 days	10 days
Dry weight of cells per vessel	19 5 mg	6 3 mg
Number of flasks mixed	6	4
Approximate volume of cells per vessel	121 mm^3	48 mm^3
Temperatures	0 9°, 5 9°, 10 9°, 15 9°, 21 0°, 28 0°	0 6°, 3 5°, 8 5°, 13 4°, 18 5°, 23 4°
Duration of experiment	47 hrs	60 hrs
CO$_2$	Not measured	Measured
Rate with 1 per cent dextrose	Not measured	Measured at 3 5° and 18 5°

50 watt Mazda bulbs, 5 per cent CO$_2$ in air was slowly bubbled through the flasks

The measurements were carried out in conical Warburg vessels shaken at about 70 oscillations per minute, a 10 per cent solution of potassium hydroxide to absorb carbon dioxide partly filling the inset in those vessels by means of which we desired to study the oxygen consumption   Six tanks at different temperatures were operated simultaneously in dark rooms, there being two experimental vessels and one thermobarometer in each for the oxygen determinations   All the vessels within one series were filled from the same suspension to make the results directly comparable   At the higher temperatures fresh air was occasionally drawn through the vessels to avoid excessive reduction of the partial pressure of oxygen

The carbon dioxide production was measured simultaneously, in Series II, by placing an extra vessel containing cell suspension but no hydroxide in each tank   Assuming the oxygen consumption within these extra vessels to be the same as in the vessels containing hydroxide, the carbon dioxide production may be determined by the formula,

$$(1) \qquad x_{CO_2} = hK_{CO} - \frac{K_{CO_2}}{K_{O_2}} x_O$$

when

x_{O_2} = mm ^{3} O_2 per hour per 2 cc of suspension measured by a vessel containing KOH,

x_{CO_2} = mm ^{3} CO per hour per 2 cc. of suspension,

K_{O_2} = Vessel constant for O_2 for the vessel *without* KOH,

K_{CO_2} = Vessel constant for CO_2 for the vessel *without* KOH,

h = mm increase in pressure per hour in vessel without KOH

The simpler formula for the determination of oxygen consumption was given in the preceding paper

III

The course of oxygen consumption as a function of time in several representative experiments at various temperatures is shown in the integral curves of Fig 1, where for each vessel the total oxygen consumption in mm ^{3} per mg dry weight is plotted against time   An inspection of the figure shows clearly that the time course of the reaction may be divided into two parts, a first portion, during which the rate is a function of time for about 25 hours, and a second, during which the rate is independent of time   Similar results are obtained for carbon dioxide production   That the change in rate during the initial phase is not due to centrifuging, or to the use of fresh Knop's solution, was proved by obtaining the same type of curve when cells in their growth medium were transferred directly to the respirometers

A simple explanation for the decline in rate exhibited by the curves in Fig 1 postulates that two substances, A and B, are oxidized during the initial phase, but only B during the final   This, of course, implies that the available supply of A is exhausted when the final rate is

exhibited   The behavior of the respiratory quotient, determined for various times at any given temperature, also suggests this, since it declines steadily from approximately 0 95 at the initial rate to the vicinity of 0 65 for the entire period of the final   Thus the ratio of
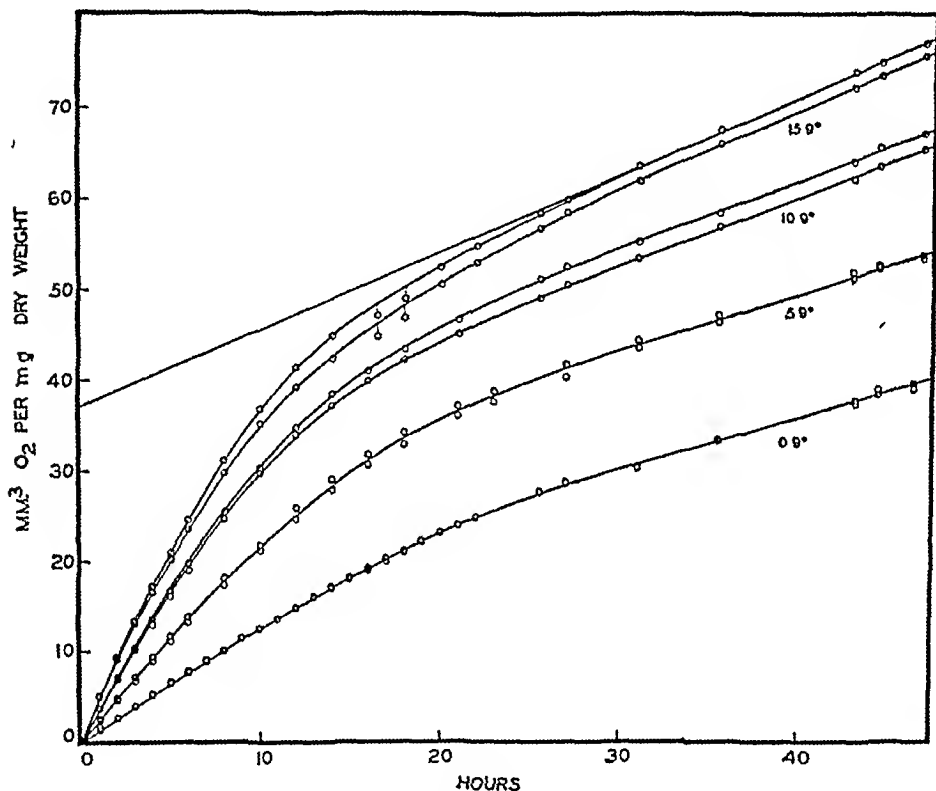

FIG 1   Typical curves obtained when total oxygen consumed, in mm ^{3} per mg dry weight, is plotted against time   Duplicate determinations are graphed for each temperature, but only at higher temperatures are separate curves drawn for each   The amount of substance A, in mm ^{3} of oxygen used by it, is given by the value of the intercept obtained when the final constant rate is projected to the y-axis at $t = 0$

respired substrates, A/B, decreases in value during the entire initial phase, when we assume A to have a respiratory quotient of 1 and B of 0 65   This assumption will be justified later   Table II gives the initial and final values of the respiratory quotient for those temperatures at which we determined the carbon dioxide production   It

appears that the initial and final values of the quotient are independent of temperature

In addition to the hypothesis that two substances are oxidized during the normal respiration of *Chlorella,* the curves of Fig 1 and the behavior of the respiratory quotient suggest that in the case of substance *A* its own concentration is the factor limiting the rate of oxygen consumption, and we may be able to describe the rate of its oxidation by some simple differential equation   If this equation be first order, the rate of oxygen consumption should be a linear function of the amount of oxygen consumed until the value of the respiratory quotient becomes constant (*i e* , until the final rate is established)   In this we

TABLE II

Initial and Final Values of the Respiratory Quotient for Chlorella pyrenoidosa at Various Temperatures

Temperature	Initial R. Q	Final R. Q
C		
0 6	0 87	0 65 ± 0 05
3 5	0 98	0 60 ± 0 04
13 4	1 00	0 70
18 5	0 94	0 65 ± 0 05
23 4*	0 93	0 70

* Additional experiment made to determine this quotient

assume that the concentration of *A* is inversely proportional to the amount of oxygen utilized

In Figs 2 and 3 the first order equation has been tested graphically by plotting the rate of oxygen consumption in mm ^{3} per vessel per hour against mm ^{3} of oxygen consumed   It will be observed that the curves at higher temperatures approximate a linear function during the initial phase, but as the temperature decreases a pronounced deviation becomes apparent   Presumably, some reaction not involving oxygen modifies the amount of *A* available for respiration, and since its effect is greater at lower temperatures, the value of the temperature coefficient for the modifying reaction must be less than that for the oxidation of *A*

Another chain of reasoning strongly suggests the presence of this

additional reaction The apparent initial concentration of A may be
measured in terms of the total amount of oxygen consumed by its
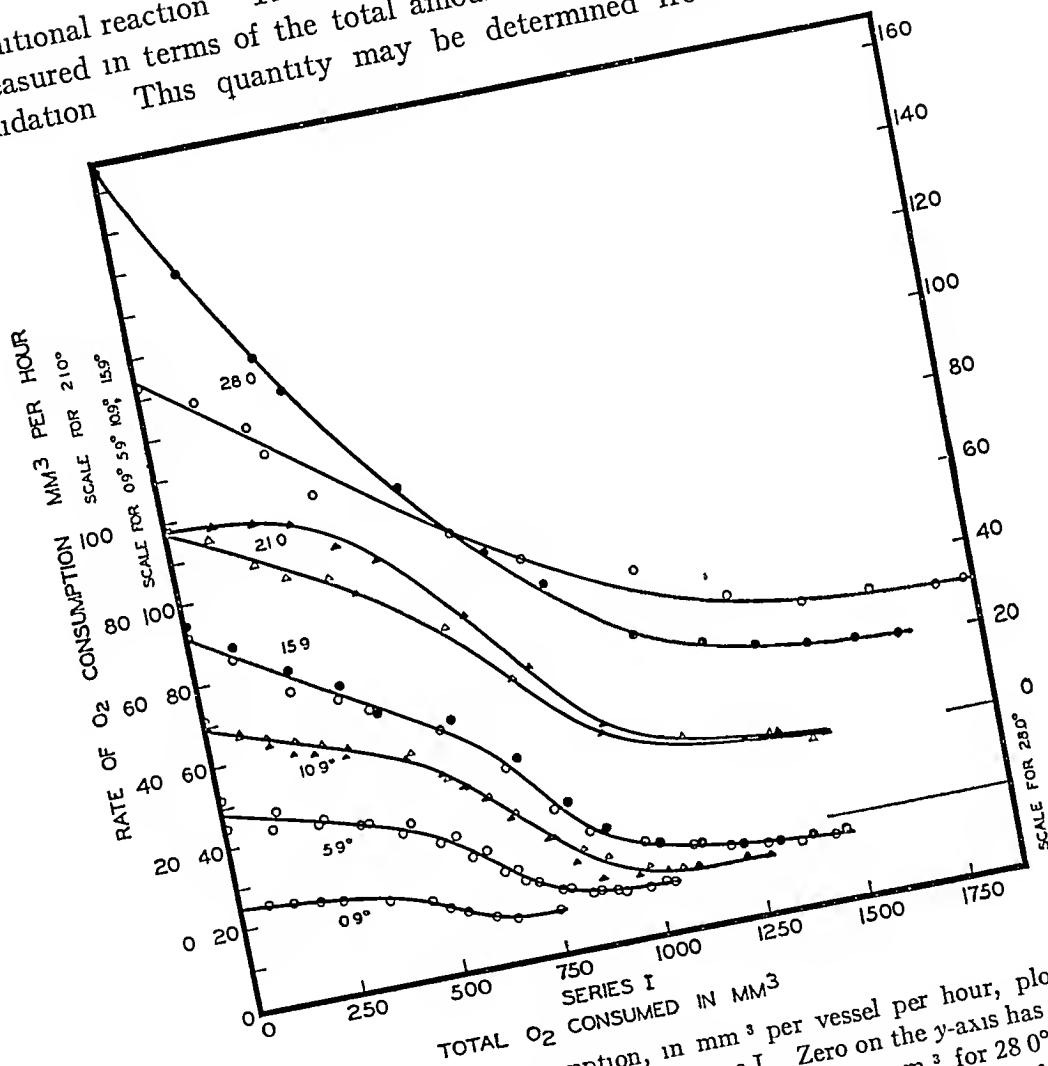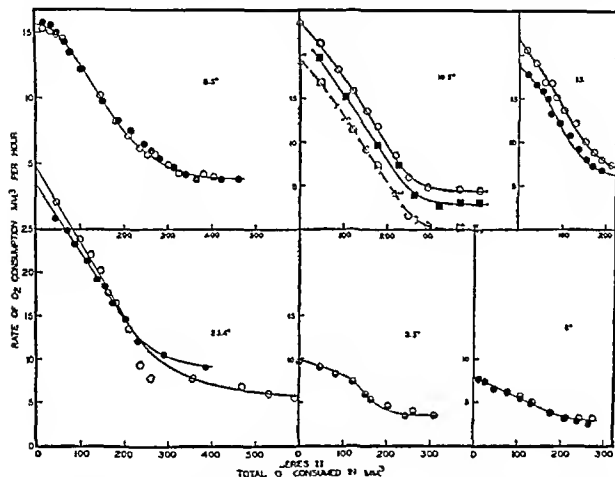oxidation This quantity may be determined from the integral

FIG 2 Rate of oxygen consumption, in mm³ per vessel per hour, plotted
against total mm³ of oxygen consumed, for Series I Zero on the y-axis has been
raised for two of the curves, that for 21 0°C is located at 20 mm³, for 28 0°C at
40 mm³ on the scale of the lower temperature curves The base lines of these
two are indicated by horizontal lines at the right

curves, some of which are shown in Fig 1, which represent the amount
of oxygen consumed as a function of time The straight line por-
tions of the curves, indicating the constant rate during the final period

due to the oxidation of substance B, are continued so as to intercept the y axis at zero time   The value of this intercept is the amount of oxygen consumed by A during the initial phase, since the extrapolation automatically subtracts the amount of oxygen consumed by the oxidation of B during this period   The results are shown in Fig 4, where

FIG 3 Rate of oxygen consumption in mm³ per vessel per hour, plotted against total mm³ of oxygen consumed, for Series II   Duplicate determinations are shown for all experimental temperatures except 18 5°C   Instead, at the latter the following are plotted against total oxygen consumed carbon dioxide production (solid squares) oxygen consumption and carbon dioxide production due to the oxidation of substance A (dotted points)

the amount of A, in terms of mm³ of oxygen used by it per mg dry weight, is plotted as a function of the Centigrade temperature   The solid circles are for Series I, the open circles for Series II   The figure states that the total amount of A is a function of temperature, which certainly cannot be so   Again, therefore, we are led to infer the existence of a reaction depleting the amount of A available for respira

tion, and since the process must have a low temperature coefficient, we suggest that it is of a simple, physical nature, such as diffusion

Previously, we have stated that the two substances, *A* and *B*, apparently involved in the normal respiration of *Chlorella* have respiratory quotients of 1 00 and 0 65 respectively   The evidence upon which this conclusion is based is derived from an analysis of the curves
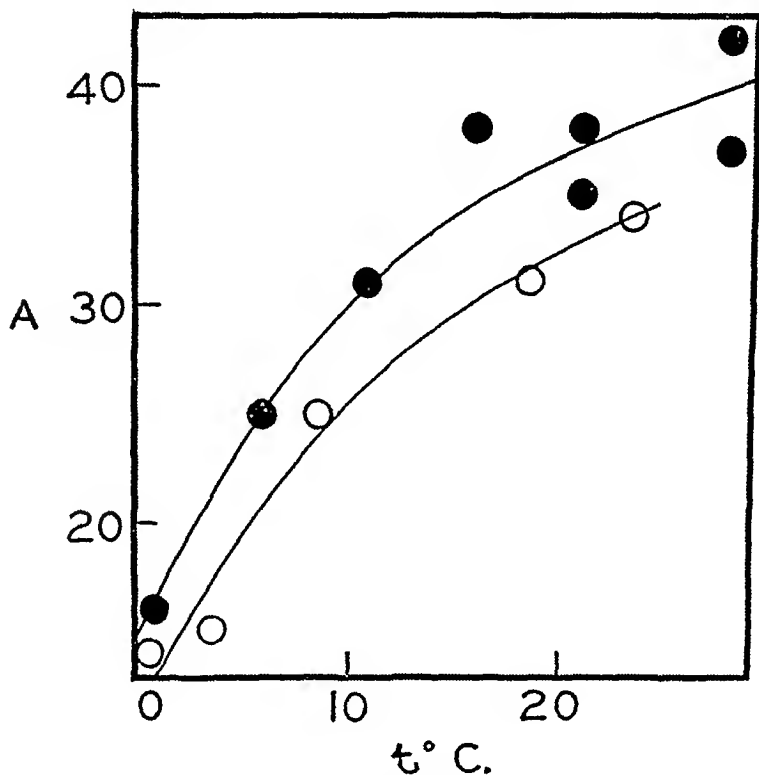

Fig 4 Apparent initial amount of substance *A*, expressed in terms of total volume of oxygen consumed by its complete oxidation, plotted against the Centigrade temperature   Solid circles indicate Series I, open ones Series II

in Figs 2 and 3, where the rate of oxygen consumption is plotted against total oxygen consumed   When the carbon dioxide data are treated likewise, curves paralleling those for oxygen are obtained, this is illustrated by the curves for 18 5°C of Series II in Fig 3, where the circles indicate oxygen and the squares carbon dioxide   In either case, if we subtract the final rate from all rates which precede it, we obtain a curve dealing only with the oxidation of *A*   This has been

done in the case of the data for 18 5°C, the resulting curve being indicated by a broken line, it will be observed that the same curve fits the corrected points for both oxygen and carbon dioxide—in other words, that the respiratory quotient is 1 00 The nearly linear relationship obtained in this case is due to the relatively high temperature, but the respiratory quotient is independent of temperature (see Table II) Since the respiratory quotient of B (0 65), determined from the final rates, likewise is independent of temperature, we conclude that two substances are used in respiration Though we cannot specifically identify them, the quotients imply that A must have the general formula, $C_x H_2 O_x$, and B must be some relatively oxygen poor substrate such as an alcohol or fat

IV

The fact that two substances may be involved in the normal, cyanide stabile respiration of *Chlorella pyrenoidosa* raises two questions first, are one or more enzyme systems involved, and secondly, can their identity be distinguished from that of the glucose respiration complex by evidence other than that from experiments involving cyanide poisoning? To answer these we offer the results of our temperature data

Experimentally it has been found that the velocity constants of chemical reactions in general vary with temperature in an orderly manner Equation 2 states this relationship

$$(2) \qquad \text{Log } K_1/K_2 = \mu/R \ (1/T_2 - 1/T_1)$$

when

K_1 = velocity constant at absolute temperature T_1
K_2 = velocity constant at absolute temperature, T
R = gas constant (1 98)
μ = constant having the dimensions of calories per degree and which has been designated variously as the energy of activation of the substrate (Arrhenius) and energy of activation of the catalyst (Rice)

Both of the above interpretations of μ agree in that each implies that the value of μ is specific for any reaction complex and relatively independent of temperature

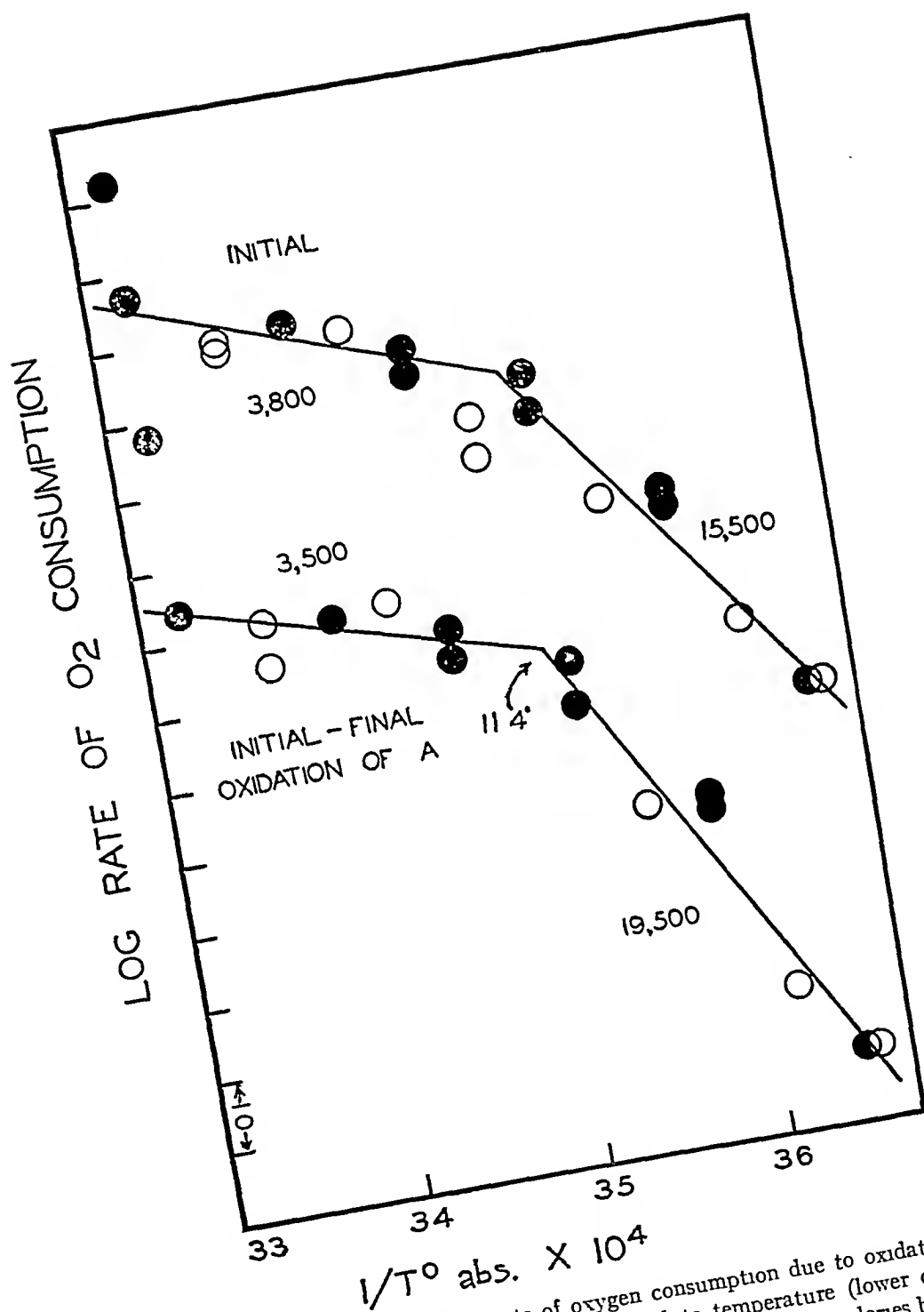In particular, Crozier (1924–25) and Crozier and Stier (1924–25,

FIG 5 Logarithm of initial rate of oxygen consumption due to oxidation of substance A plotted against reciprocal of absolute temperature (lower curve) For this process μ has a value of 3,500 calories above and 19,500 calories below a critical temperature near 11 5°C For comparison, the plot for the initial rate (due to oxidation of A plus B) is shown (upper curve) Solid circles represent Series I, open circles Series II

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1926–27) have applied this equation to a considerable array of biological data in order to determine whether μ is a constant such as may be used to characterize a given physiological process   To determine μ, the natural logarithm of the rate is plotted against the reciprocal of the absolute temperature, and if a straight line may be drawn through the resulting points, the value of its slope is $- \mu/R$ On occasion
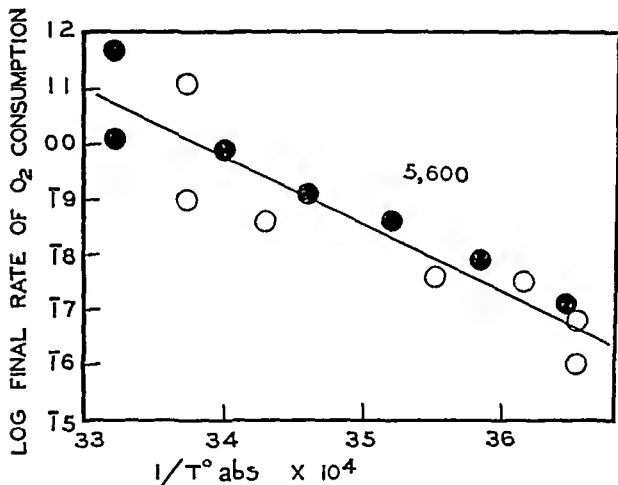
FIG 6 Logarithm of final rate of oxygen consumption plotted against reciprocal of absolute temperature   The value of μ is 5 600 calor es and is related to the oxidation of substance B Solid circles for Series I, open circles for Series II

it is necessary to draw two straight lines of different slopes for two temperature ranges through the experimental points   It is of interest to determine the values of μ for the oxidation of A and B, and to compare these with the values obtained for the glucose respiration and for the respiration of other organisms

In Fig 5 there are plotted the natural logarithms of the initial rate (rate of A plus B) of oxygen consumption and the initial rate for A

against the reciprocal of the absolute temperature   The former has been plotted merely for purposes of comparison, since its value of μ, depending upon the sum of two rates, is without theoretical significance   Unless we wish to fit the data with a curved line, the initial rate has two values of μ, 3,800 above and about 15,500 below a critical temperature in the neighborhood of 11 5°C   The initial rate of oxygen consumption by A gives μ values of 3,500 above and 19,500 below the critical temperature   Since the respiratory quotient does not vary with temperature, the value of μ for carbon dioxide production will be identical   The observations of Yabusoe (1924–25) when so plotted gives μ the value of 9,500 from 10 to 30°C   This is on the basis of determinations at but three different temperatures, however, and was made on *Chlorella vulgaris* instead of *pyrenoidosa*

TABLE III

Rate of Oxygen Consumption by Chlorella with and without Glucose

Temperature	Glucose	Normal	$\frac{Normal}{Glucose}$
°C			
3 5°	1 95	1 5	1 3
18 5°	8 9	4 3	2 0

The μ plots for the final rates of oxygen consumption, which represent the rate of oxidation of B, are given in Fig 6, where the solid circles represent Series I and the open circles Series II   The value of μ, 5,600, is constant over the entire temperature range, and applies to carbon dioxide production as well, since the final respiratory quotient is also independent of temperature

As a check on previous experiments in which the glucose respiration of *Chlorella* was investigated, the rate of oxygen consumption in Knop's solution containing 1 per cent glucose was determined   The results at two temperatures, 3 5° and 18 5°C , are given in Table III   On the basis of these two points the value of μ is 19,000 calories, which agrees with that published previously

In Fig 7 the observed initial and final rates of oxygen utilization are plotted directly against the temperature   It is of interest that, in

data with this degree of scatter, the rates appear to be linearly related to the temperature

V

Table IV summarizes values of μ obtained for several metabolic processes in *Chlorella pyrenoidosa*, and indicates that different processes
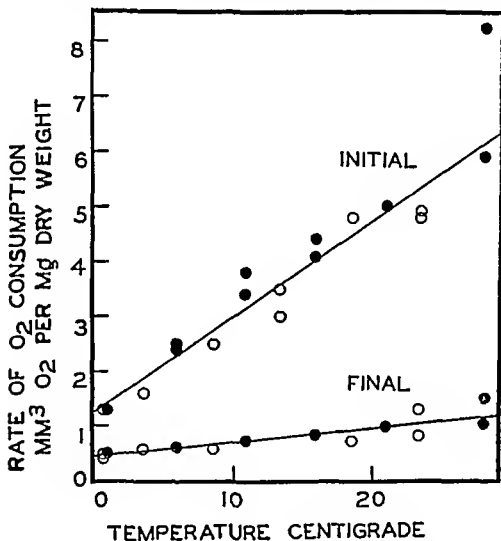

Fig 7 The initial and final rates of O_2 consumption when plotted against temperature Centigrade give a straight line   Compare with Figs 5 and 6 and see text.

within the same organism may be characterized by different values of this constant   The values of μ for the different types of respiration not only differ among themselves, but from that for photosynthesis as well   Since the values for the respiration of A and B differ, we have further evidence favoring the idea that the normal respiration of

Chlorella involves at least two different systems   For the determination of values of μ it is not wise to assume that the quantity measured is dependent upon a single reaction complex, unless an attempt has been made to check this assumption experimentally   In the case of our experiments, this would have meant the acceptance of the value of μ determined on the basis of the initial rate (*A* plus *B*), a value dependent upon the rates of two processes

The value of μ obtained for the respiration of *A* below the critical temperature agrees with that for the glucose respiration (19,000),

TABLE IV

Values of μ Determined for Metabolic Processes in Chlorella pyrenoidosa

Process	μ		Critical temperature
	Above critical temperature	Below critical temperature	°C
Oxidation of *A*	3,500	19,500	11 5
Oxidation of *B*	5,600		
Oxidation of *A* plus *B* (initial rate)	3,800	15,500	11 5
Photosynthesis	13,000 ± 1,000†		
Glucose oxidation	(12,000)	19,000	(15)
H_2O_2* decomposition by intact cells	10,500		

* French (1934–35)
† Unpublished data of W A Arnold and H I Kohn

whereas above the critical temperature, it does not (3,500)   The 19,000 value has been obtained for yeast by Stier (1932–33) between 3–15°C , and by Lineweaver, Burk, and Horner (1931–32) for *Azotobacter*

The values of μ obtained for the oxidation of *A* above the critical temperature (3,500) and for the oxidation of *B* throughout the experimental temperature range (5,600) are the lowest yet recorded for a respiratory process   In part, this may be due to the fact that in temperature studies of respiration this process has not been sufficiently separated out from other concomitant processes

SUMMARY

The respiration of the green alga *Chlorella pyrenoidosa*, suspended in Knop's solution, has been studied in the dark as a function of time and of temperature   The rates of oxygen consumption and of carbon dioxide production (at constant temperature) decline for about 25 hours to a low, constant level   From an analysis of the curves it is suggested that two substances, A and B, are utilized, whose respiratory quotients are 1 and 0 65 respectively   The values of the temperature characteristics were found to be for oxidation of A, 19,500 (0 6 to 11 5°C) and 3,500 (11 5 to 28°C), for oxidation of B, 5,600 (23 4 to 0 6°C)

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TEMPERATURE CHARACTERISTICS FOR THE METABOLISM OF CHLORELLA

III THE CATALYTIC DECOMPOSITION OF HYDROGEN PEROXIDE BY CHLORELLA PYRENOIDOSA

By C S FRENCH

(From the Laboratory of General Physiology, Harvard University, Cambridge)

I

As some suggested mechanisms for cellular respiration postulate the formation of hydrogen peroxide as a by product, the study of its decomposition by living cells is of some general interest   It was suggested by Warburg and Uyesugi (1923–24) that the same enzyme system is responsible for both peroxide splitting and the Blackman reaction of photosynthesis

Yabusoe (1924–25) measured this reaction at different temperatures, using *Chlorella vulgaris*   He gives a straight line relation between rate and temperature Centigrade from 10 to 30°C, the 5° point being out of line with the others   If his data are used to test the Arrhenius equation by plotting the logarithm of the rate against the reciprocal of the absolute temperature it fits equally well except that the 30° point is too low   As disturbing effects of higher temperatures often occur, it seems more sensible to present his data in this form   The value of μ calculated from his points at 5°, 10°, and 20° is 17,500   A second experiment with points at 10° and 20°, however, gives $\mu = 12,000$, the 30° points were disregarded in both sets for this calculation

To compare μ for hydrogen peroxide splitting with that for other processes in *Chlorella pyrenoidosa* (Crozier, Tang, and French, 1934–35, French, Kohn, and Tang, 1934–35) it was decided to make similar measurements on this species and to confine the work to temperatures below 20°

II

A large bottle was sterilized and half filled with 4 liters of sterile Knop's solution   It was inoculated with 150 ml of *Chlorella* suspension grown in flasks in the usual way (Crozier, Tang, and French, 1934–35)   The bottle was placed in the incubator used for the flasks, and in addition was illuminated from one side by a 500 watt projection bulb focused evenly over the whole side   Between the culture bottle and the light there was a heat screen of 4 7 cm dilute ferrous ammonium sulfate with a little sulfuric acid   A stream of cold water through the incubator held the temperature at about 20°   After 8 days of growth with the usual mixture of 5 per cent CO_2 in air bubbled through, the culture was centrifuged and the cells were rinsed once with 35 ml. phosphate buffer of pH 6 72 and resuspended in 50 ml of the buffer solution   This suspension was kept on ice during the day and a half required for the experiment   Each ml contained 80 mm ^{3} of cells   A solution of H_2O_2 was made up of Merck superoxol in the same buffer

The reaction mixture consisted of 25 ml of the peroxide solution with which, after 15 minutes for thermal adaptation in separate flasks, was mixed 4 ml of the cell suspension, thus making a 0 0238 M H_2O_2 solution containing 11 mm ^{3} of cells per ml in M/15 phosphate buffer of pH 6 72   5 00 ml was pipetted for titration into a flask containing 100 ml water, 15 ml H_2SO_4 (1 2), and 5 ml 20 per cent KI   Other samples were taken, at various time intervals, using the same pipette which had been washed and dried

Immediately after the sample was pipetted into the titration mixture it was warmed for exactly 2 minutes on an electric hot plate, bringing it to 44°, then allowed to stand covered for 2 more minutes before the liberated iodine was titrated with $Na_2S_2O_3$ solution   Unless the solution is treated in this way the reaction is not complete, and if allowed to stand too long there is danger of loss of iodine   The first sample was not considered reliable due to gas bubbles in the mixture, so the initial concentration of H_2O_2 was determined by titrating a 5 ml sample of H_2O_2 solution which had been mixed with 4 ml of buffer instead of with cell suspension   Separate determinations gave 15 66 and 15 73 ml thiosulfate *to* combine with the liberated iodine

III

Experiments were made at 0 6°, 4 4°, 10 9°, 15 2°, and 20 0° The time course of the reaction was followed at each temperature by taking samples at various intervals
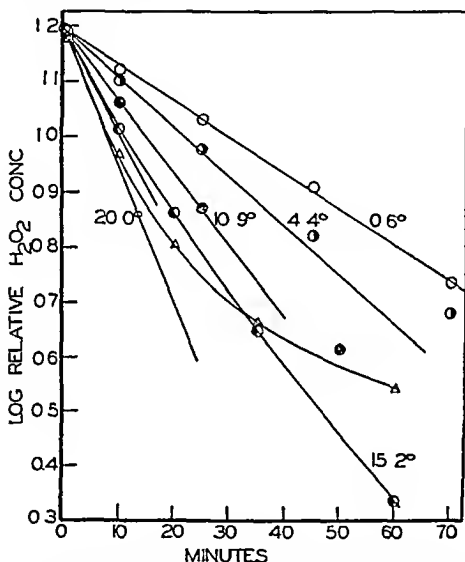

FIG 1 The logarithms of the hydrogen peroxide concentrations (expressed in ml. of $Na_2S_2O_3$ equivalent to it) in suspensions of *Chlorella* cells are plotted against time for experiments at five temperatures The straight lines represent the initial slopes of the curves

At the lowest temperature the data are accurately fitted by a first order velocity equation, from which, however, large deviations are found at higher temperatures This equation is useful in approximating the initial rates in which we are interested Fig 1 shows the logarithm of the number of ml of 0 0151 N $Na_2S_2O_3$ equivalent to the

liberated iodine plotted against time, for the various temperatures
Curves are drawn through the points and the slope of the curve at
$t = 0$ is taken as a measure of initial velocity   The destruction of the
active enzyme during the reaction does not influence the initial rates
If the first order equation is obeyed the curve would be straight, since
the first order equation

$$K = 1/t \log \left(\frac{a}{a - x} \right)$$
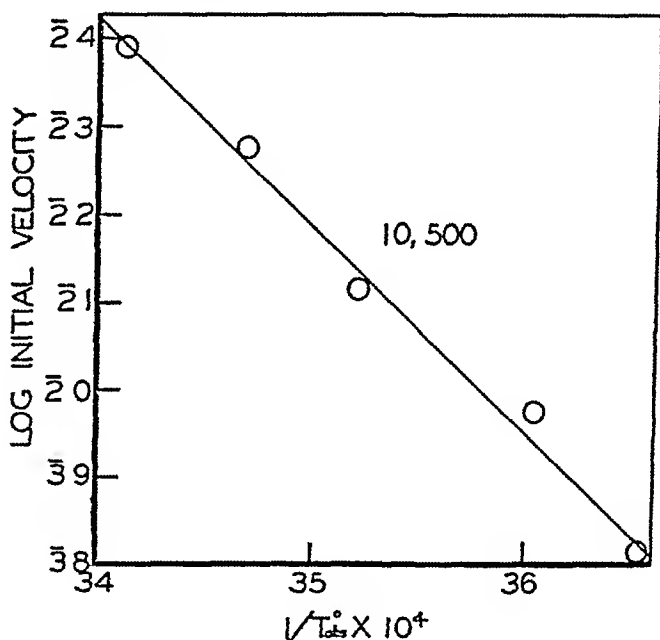
may be written

$$\log (a - x) = \log a - Kt$$

FIG 2   The logarithms of the initial rates of disappearance of added hydrogen
peroxide in suspensions of *Chlorella* are plotted against reciprocal absolute tem-
perature   The line corresponds to a value of $\mu = 10,500$ calories

which is the equation of a straight line relation between $\log (a-x)$ and
t, a is the initial H_2O_2 concentration and $(a-x)$ is the concentration at
time t, these are expressed directly in ml of thiosulfate   The loga-
rithms of the initial rates of H_2O_2 decomposition are plotted against
reciprocal absolute temperature in Fig 2   They are fitted by a
straight line with a slope of $\mu = 10,500$

IV

Since the results obtained at the higher temperatures fall off considerably from first order curves, we may conclude that the substance responsible for the catalytic destruction of H O is decomposed during the reaction This is in accord with the usual kinetics of catalase (Williams, 1927–28, and Yamasaki, 1920) and indicates that we are dealing with an enzyme rather than with a stable surface action that might well be the cause of decomposition of $H O_2$ by intact cells Sohngen and Smith (1924), however, find that for yeast cells the first order equation is followed at temperatures between 25–50°C It may be that in their case the action was due to the cell wall or other stable constituents of the protoplasm Their results give $\mu \approx 12,200$ from 25–50°, substantially in agreement with this determination for *Chlorella*

The value of μ here found, 10,500, is quite different from either the values 3500 or 19,000 found previously for respiration of *Chlorella* without added glucose and 19,000 for O consumption in 1 per cent glucose solution It is seen that various processes in the same organism need not necessarily behave the same way in relation to temperature The interesting possibility has been suggested by Crozier that this value of μ might be changed by the presence of glucose or other substances in solution The effect of light on the reaction is also unexplored

SUMMARY

The decomposition of hydrogen peroxide by intact *Chlorella* cells follows a first order course at very low temperatures, but at higher temperatures gives falling first order constants Between 0 6° and 20°C the value of μ is 10,500 calories

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NATURE OF THE ACTION CURRENT IN NITELLA

I. General Considerations

By W. J. V. OSTERHOUT

(From the Laboratories of The Rockefeller Institute for Medical Research)

It has been suggested[1] by Blinks and by the writer that potassium[2] plays an important rôle in the action current of *Nitella* Whether the outstanding features of the action curve can be explained in this way is a matter of considerable interest

An experimental basis for such an explanation is found in observations by Damon[3] who states that marked changes in P.D. may be caused by the movement of potassium ions He found that in sea water *Valonia* showed an inwardly directed (negative) P D of 8 mv [4]

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[1] Blinks L R, *J Gen Physiol*, 1929–30, 13, 495 Osterhout W J V, Harvey Lectures 1929–30 25 169, *Biol Rev*, 1931, 6, 369, *Ergebn Physiol* 1933, 35, 967

[2] What is said in this paper regarding potassium applies in lesser degree to the other cations present but their effect is relatively small The special rôle of K^+ may be due to its high mobility The mobility of H^+ is probably high but it has little effect owing to its low concentration Cf Osterhout, W J V, *J Gen Physiol*, 1929–30, 13, 715 Organic ions in W may play a part in producing the resting potential which in some cases is too large to be accounted for by K^+ alone Such an effect is possible on account of the difference between λ and l (see Osterhout W J V, *Ergebn Physiol*, 1933, 35, 1013)

[3] These experiments were made on *Valonia macrophysa*, Kütz Cf Damon, E B *J Gen Physiol* 1929–30 13, 207 1931–32 15, 525 (in which papers positive potential denotes what is called negative in the present article where a potential is regarded as negative when the positive current tends to flow from the external solution across the protoplasm to the sap and in the external circuit from the capillary to the external solution)

A variety of curves was obtained the graph shown in Fig 1 was selected as showing clearly certain features of importance in the present discussion

[4] It is not necessary to discuss this in detail but the following points may be of interest The sap contains about 0 5 M KCl which might be expected to give an outwardly directed potential, but there are probably substances in the aqueous

but in artificial sap, containing 0 5 M KCl, this rose[5] to 60 mv., after which it fell off

This falling off (shown by the rise of the curve in Fig 1) may be interpreted as the effect of the entrance of K^+, penetrating as a more or less well defined moving boundary[6]   In Fig 1 the symbol K is employed to denote K^+ penetrating from without (as the changes in P D are wholly due to the K^+ which penetrates from without the K^+ present in the cell at the start is not represented)

As in previous papers[7] it is assumed that the protoplasm consists of three layers the outer surface X and the inner surface Y (both non-aqueous) between which is an aqueous layer W In *Valonia* the concentration of potassium in all of these layers[8] is probably low at the start

When potassium is applied to the outer surface of *Valonia* (Diagram A of Fig 1) it raises the negative potential to 60 mv   but after it has entered and attained the same concentration inside X as outside (Diagram B) this effect disappears so that the P D returns to the neighborhood of 8 mv[9]   Presumably the potassium when it reaches

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layer (W) of the protoplasm which set up different P D's with X and Y, as the result of which the total P D is 8 mv negative   It has been shown that X and Y differ in several respects (*cf* Osterhout, W J V, *Ergebn Physiol*, 1933, **35**, 1013)

[5] *Cf* Damon, E B, *J Gen Physiol*, 1932–33, **16**, 375

[6] Regarding moving boundaries see MacInnes, D A, and Longsworth, L G, *Chem Rev*, 1932, **11**, 171   The fact that very little dissociation may occur in the non-aqueous surface layers X and Y makes no essential difference in this slow process (as ions pass out of Y more will be formed by dissociation of the molecules present in Y)   *Cf* Osterhout, W J V, *Ergebn Physiol*, 1933, **35**, 1009

[7] *Cf* Osterhout, W J V, *J Gen Physiol*, 1927–28, **11**, 83, *Bull Nat Research Council, No 69*, 1929, 170, *Ergebn Physiol*, 1933, **35**, 1013

[8] The fact that potassium accumulates in the vacuole, reaching a concentration of 0 5 M, does not mean that it accumulates in the protoplasm, which may conceivably have as low a concentration as the sea water, *i e* about 0 012 M This would be expected if its pH were as high as that of the sea water (*cf* Osterhout, W J V, *Ergebn Physiol*, 1933, **35**, 982)

[9] The values given in this paper are obtained by assuming that the potentials at the liquid junctions of the two calomel electrodes saturated with KCl are approximately equal and hence so nearly cancel out that they may be neglected This is the assumption commonly met with in the literature but the basis on which it is made may be questionable   It may be noted that on the basis of

Y (Diagram C) sets up a negative potential which is partially lost when it reaches the inside of Y (Diagram D) [10] Discussion of the subsequent fall of the curve is omitted since it seems to have no bearing on the fall of the action curve of *Nitella*

Let us now consider the action current of *Nitella* (Fig 2) as its movements, o, p, and q, resemble those in Fig 1 we may inquire whether they can be due to movements of K^+ If so K^+ must move outward[11] from the sap (where its concentration is relatively high[12]) toward the external solution (where its concentration is low[13]) and such movement should be brought about by applying an electric current carrying K^+ outward but not by one in the opposite direction This agrees with the facts (According to the local circuit theory stimulation occurs only as the result of an outgoing current[14] and the stimulus is propagated along the cell by a series of changes which set up such a current at each spot in turn)

The P D at the start is presumably due chiefly to K^+ in the sap,

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this assumption we arrive at measurements of the membrane potential in the Donnan equilibrium which agree closely with the calculated values These calculations for dilute solutions where the activity coefficient is approximately unity must be approximately correct

[10] It may seem surprising that 0.5 M K^+ diffusing inward should raise the concentration of K^+ just inside Y where it is already 0.5 M, but in reality it is to be expected since experiments have shown that raising the external concentration of potassium causes the internal concentration to increase (*cf* Jacques A G, and Osterhout, W J V, *J Gen Physiol*, 1931–32, 15, 537) It is probable that the concentration of K^+ is always higher just inside Y than just outside (*cf* footnote 8)

[11] Hence the movement o is in the negative direction instead of in the positive direction, as in Fig 1 In case part of the resting P D is due to organic ions in W these must also move or alter their charges in order to reduce the P D to zero

[12] The sap contains about 0.05 M KCl and about 0.05 M NaCl, together with other substances

[13] In the experiments where action currents occur the concentration of K^+ in the external solution is usually 0.001 M or less

[14] Stimulation may occur when an ingoing current is broken but in this case an outgoing current, which occurs immediately after the break (due to polarization) is believed to be responsible (regarding polarization see Bbnks L R., *J Gen Physiol* 1929–30 13, 495)
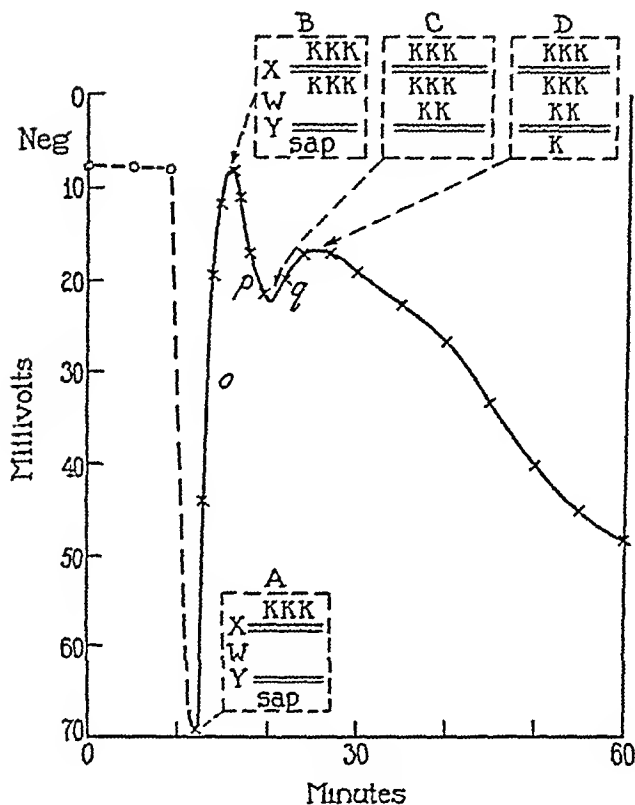
FIG 1 Shows changes in the P D of *Valonia*   The broken line shows the negative P D found in sea water (symbol O) and its increase when potassium is applied to the exterior   The unbroken line shows subsequent changes supposedly due to the penetration of potassium   Observations made after the application of potassium are denoted by the symbol X

In the diagrams the symbol K denotes the penetrating potassium   (reduction in concentration is shown by reduction in the number of symbols, *i e*, KK means a lower concentration than KKK)   Each stage of its progress is marked by a change in P D   for example, in Diagram A the observed P D is supposedly due to the relatively high concentration of potassium at the outer surface of X, in Diagram B we see that potassium has reached the inner surface of X and in consequence most of the P D has disappeared
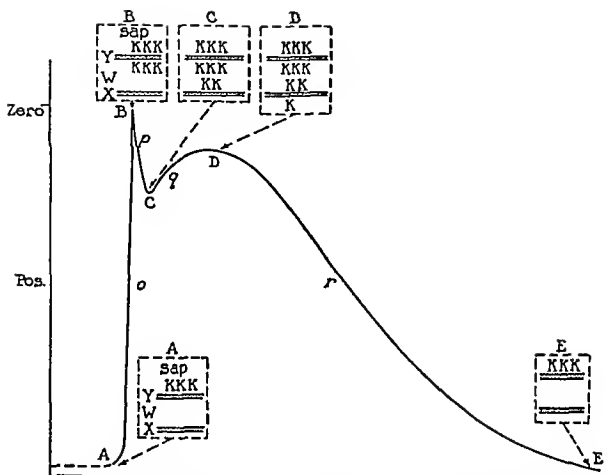
Fig 2 The unbroken line shows changes in P D during the action current in *Nitella* supposedly due to the outward movement of potassium The broken line shows the P D in the resting state, before the outward movement of potassium begins

In the diagrams the symbol K denotes the outwardly moving potassium (reduction in concentration is shown by reduction in the number of symbols) Each stage of its progress is marked by a change in P D for example, in Diagram A the observed P D is due to the relatively high concentration of potassium at the inner surface of Y, in Diagram B we see that potassium has reached the outer surface of Y and in consequence the P D has disappeared

The duration of the action current is usually about 15 seconds.

causing an outwardly directed (positive) potential at Y [15] The supposed movement of potassium is illustrated by Fig 2 where the symbol K is used to denote K^+ which is present in the sap at the start and which moves outward during the negative variation (as previous experiments indicate that the concentration of K^+ in the protoplasm at the start is usually small it will be regarded as negligible)

It seems probable that the outward movement of K^+ is facilitated by an increase in the permeability of Y since otherwise it would be more gradual [16] In order to bring about an abrupt loss[17] of P D (Fig 2) by the movement of K^+ alone K^+ would have to advance in

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[15] This might possibly be questioned in view of the fact that in *Halicystis* and in *Valonia* Y does not appear to give much potential with K^+ This is shown in *Halicystis* by perfusion experiments in which K^+ is introduced into the vacuole (*cf* Blinks, L R , *J Gen Physiol* , 1934–35, **18**, in press) and is rendered questionable in *Valonia* by the fact that in spite of the presence of 0 5 M KCl in the vacuole the potential across the protoplasm is inwardly directed (negative) But in *Valonia* there may be a high positive potential at Y due to K^+ which is compensated by potentials due to organic substances in W (*cf* Osterhout, W J V , *Ergebn Physiol* , 1933, **35**, 1014) and producing unlike effects on X and Y

In *Nitella* the situation is quite different Here X and Y are much more alike This is shown by the fact that when sap is applied to the outside of the cell, giving the chain

$$\text{sap} \ \bigg| \ \begin{array}{c} \text{protoplasm} \\ X \mid W \mid Y \end{array} \ \bigg| \ \text{sap}$$
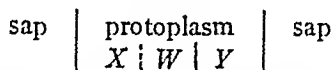
we obtain 15 mv or less whereas in *Halicystis* we get 50 mv and in *Valonia* 65 mv (Osterhout, W J V , *Ergebn Physiol* , 1933, **35**, 1014) If X and Y are not very dissimilar we must conclude that Y gives a high potential with K^+ since we know this to be the case with X

[16] As, for example, in *Valonia* (*cf* Fig 1 where the times are much greater than in Fig 2)

[17] In *Nitella* the o movement appears as a rule to consist essentially of a loss of the P D across the protoplasm but as will be shown in a later paper it depends to some extent on other factors It may be noted that in nerve, according to Gasser (Gasser, H S , *Am J Physiol* , 1931, **97**, 254, Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, Long Island Biological Association, 1933, **1**, 138) temperature has more effect on the negative variation than on the resting P D , which suggests that the former involves something more than a mere loss of the latter Bishop finds that certain anesthetics abolish irritability without producing much effect on the resting potential (Bishop, G H , *J Cell and Comp Physiol* , 1932, **1**, 177)

the form of a sharply defined moving boundary with a concentration of K^+ equal to that in the sap so that as soon as it emerged into W the concentration of K^+ would become the same on both sides of Y This seems improbable since Y appears to be non aqueous,[18] with a low dielectric constant and hence not permitting much dissociation Consequently the concentration of K^+ must be much lower in Y than in the sap and the front of the moving boundary must have a correspondingly low concentration unless an increase occurs in the permeability of Y to K^+

An increase in permeability is assumed by most writers in discussing action currents Such an increase might occur if during the outward flow of current Y suffered a breakdown or rupture[19] or such a change in composition as to admit K^+ at the concentration found in the sap [20] Breakdown or rupture could doubtless be brought about by the electrical gradients present in the cell which might conceivably amount to more than 20,000 volts per millimeter[21] A change in composition might occur if the current carried substances from the sap into Y [22]

Such changes might make it possible for K^+ to travel outward in the form of a moving boundary, having a concentration of K^+ which is not very different from that in the sap We should therefore expect the o movement to be sudden[22] and if the alteration of Y consisted of

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[18] Osterhout, W J V, *Ergebn Physiol* 1933, **35**, 1009

[19] A rupture appears to occur in the case of mechanical stimulation (*cf* Osterhout W J V and Hill S E, *J Gen Physiol*, 1930–31, **14**, 473) and in the case of stimulation by cold (Hill, S E *J Gen Physiol*, 1934–35, **18**, 10 press)

[0] This would involve a rise in the dielectric constant

[21] The P D across the protoplasm of *Nitella* (in contact with pond water) is 100 to 300 mv and Y may be only a few molecules thick if it were 0 01 of a micron in thickness the drop might be 20,000 volts per mm

[22] A substance is supposedly carried from the sap into Y when the potassium effect is restored by an action current (*cf* Osterhout W J V, and Hill S E,

breakdown or of rupture there could be a sudden loss of P D even before much movement of K⁺ had taken place

Should the increase in permeability fail to occur we should expect a failure of stimulation, such as is found, for example, in cells leached in distilled water until they have lost their irritability [24]   Here the P D across the protoplasm is not less than normal[25] so that when an outgoing electrical current is imposed from an external source we should expect stimulation unless the increase in permeability were prevented by an unusual condition of the non-aqueous layers[26] due to the treatment   Such cells are found out of doors at certain seasons

Let us now consider the next movement (p, Fig 2) which is supposed to result from the fact that outwardly moving K⁺ comes in contact with X and sets up an outwardly directed (positive) P D similar to that found at Y in the resting condition of the cell   Since little or no potential remains at the end of the o movement K⁺ is no longer carried outward by an electrical current but moves by diffusion aided by the continual agitation of W which results from the vigorous motion of protoplasm and sap which is characteristic of these cells [27] Hence the p movement is slower than the o movement, sometimes very much so

As K⁺ continues its outward movement and reaches the outer side of X it will cause the P D to fall off again (q, Fig 2)   As X is presumably a non-aqueous layer, the amount of K⁺ passing through it will be correspondingly small and as a result we may expect the q movement to be relatively slow, especially as the moving boundary will tend to lose sharpness in W owing to agitation by protoplasmic movement   This agrees with observation

[24] Cf Osterhout, W J V , and Hill, S E , *J Gen Physiol*, 1933–34, **17**, 87

[25] This situation recalls that in a nerve treated with cocaine

[26] The treatment with distilled water removes the potassium effect from X but not from Y for the outwardly directed potential of the resting state is presumably due chiefly to the effect of K⁺ on Y and this remains after treatment with distilled water   But it removes from Y the ability to undergo an increase of permeability due to an outgoing current

[27] According to François-Franck and Auger (François-Franck, L , and Auger, D , *Ann physiol et physicochim biol*, 1933, **9**, 983) the protoplasmic motion slows down within 2 seconds after the passage of a negative variation   Similar observations were made by Hormann (Hormann, G , Studien uber die Protoplasmastromung bei den Characeen, Jena, Gustav Fischer, 1898)

When K^+ has reached the outside of X we should expect the cell to behave somewhat like a resting cell to which KCl is applied externally This is the case according to Blinks [8] who states that polarization to either an ingoing or an outgoing current now falls off on stimulation just as when the cell is placed in contact with 0 1 M KCl [9]

The final fall of the curve (r, Fig 2) may be regarded as a process of recovery[30] by which the potassium is gradually moved back into the sap as the result of (1) the ingoing electric current which occurs during recovery according to the local circuit theory,[31] and (2) the forces which (even in the absence of current) cause potassium to move into the cell and accumulate there The speed of recovery falls off in such fashion as to indicate that it is primarily a process of diffusion

According to this hypothesis the double peak of the action curve is due to the presence of two protoplasmic surfaces X and Y both of which give a relatively high potential with potassium It follows that if X were insensitive to the action of potassium we should get only a single peak This is apparently the case with Chara [32] The experiments of Blinks[33] show that in Chara the outer surface is not more

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[8] Blinks L R J Cen Physiol 1929-30 13, 495 Blinks finds that an ingoing electric current (passing from the external solution to the sap) produces no polarization when 0 1 M KCl is applied externally This might be explained by saying that the protoplasmic surface is very permeable to K^+ (and for the same reason no polarization is produced by an outgoing current which carries K^+ outward from the vacuole) But there are limits to this since Blinks finds that a very large ingoing current does produce considerable polarization when 0 1 M KCl is applied externally This is formally analogous to the polarization of a non polarizable electrode when the current exceeds a certain value although the mechanism differs

[9] There is other evidence that changes in the properties of the protoplasm can be produced in Vitella by potassium Hober (Hober R Physikalische Cheme der Zelle und der Gewebe Leipsic Wilhelm Engelmann 6th edition, 1926 641 785) has suggested this for nerve and muscle

[30] This is not comparable to the fall of the Valonia curve (Fig 1) which does not seem to be attributable to recovery

[31] Cf Osterhout W J V Harvey Lectures 1929-30 25 169 Biol Rev 1931 6, 369

[32] The species used was Chara coronata Ziz

[33] Blinks L R Proc Soc Exp Biol and Med 1932 33 30 756

sensitive to potassium than to sodium [34]   In *Chara* the action current has only one peak (Fig 3), as would be expected

This explanation of the action curve seems to be useful as a working hypothesis since it accounts not only for normal behavior but also for the chief deviations, some of which will be discussed in subsequent papers   But we cannot decide about its correctness without further facts   One objection to it has been discussed by Blinks [28]   This is that recovery can occur while an outgoing electric current is being
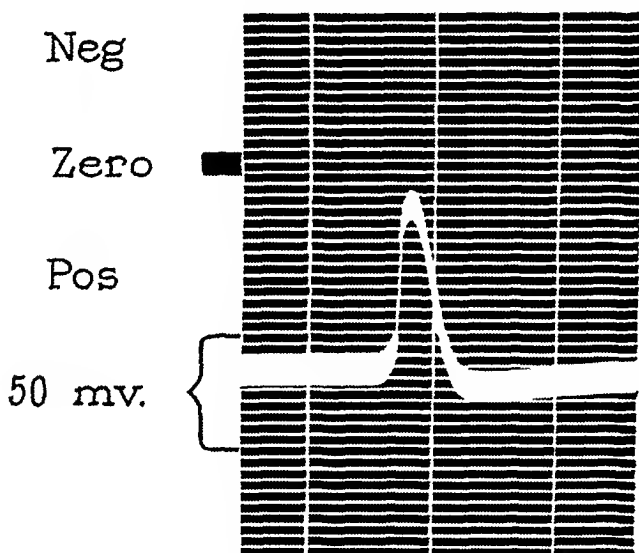
FIG 3 Photographic record showing the action curve of *Chara coronata*, Ziz The curve has a single peak which may be due to the fact that only the inner protoplasmic surface gives a large electrical effect with potassium (with the outer surface potassium acts much like sodium)   The right end of the cell was killed with CHCl₃   The spot at which the action current was recorded was in contact with 0 001 M KCl   On stimulation the P D dropped to zero   Vertical lines are 5 seconds apart   Temperature 24°C

applied from an external source   It is possible that this may be due to changes in permeability   Although at first the outward current appears to make *V* more permeable to K^+ it would not be surprising if, as the current continued to flow, this effect should diminish as the

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[34] In *Nitella* 0 01 M KCl gives about 80 mv against 0 01 M NaCl (the KCl being negative), but in *Chara* the value is very small

result of the movement of substances[35] or of changes in hydrogen ion concentration (as in the experiments of Bethe and Toropoff[36]) or of other alterations in metabolism   If, as a result, the permeability of Y to K^+ should diminish somewhat[37] while the current is flowing outward the concentration of K^+ would increase just inside Y and diminish just outside Y This would produce an outwardly directed (positive) potential which would increase until equal to that in the resting state, thus producing recovery   It might go further and produce a greater positive potential, resembling the "after potential" found in nerve [38]

With such a flow of current from an external source or with an action current "after potential" (which seldom occurs in *Nitella*) might arise in several ways, e g (1) as the result of a temporary increase in the concentration of K^+ just inside Y due to a change in permeability, as just mentioned, (2) as the result of a temporary increase in pH value in W or just outside X which would increase the gradient of KOH across Y and cause potassium to move inward more rapidly and reach a higher concentration just inside Y than usual [39]   That this might happen is indicated by the experiments of Bethe[40] and of Blinks [41] (3) The movement of organic cations into W as the result of the action current   These might increase the positive potential by having different effects on Y and X Such substances are apparently responsible for some of the positive P.D in *Nitella* and for a good deal of the positive P D in *Halicystis* [42]   Such substances might not persist long in

[35] Such a movement appears to change λ when an action current restores the potassium effect according to Osterhout and Hill unpublished results

[36] Bethe, A , and Toropoff, T *Z phys Chem* , 1914 88, 686, 1915, 89, 597

[37] This change need not be sufficient to affect the flow of current greatly

[38] Gasser H S , *Am J Physiol* , 1931, 97, 254, Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, Long Island Biological Association, 1933, 1, 138

[39] Osterhout, W J V , *Ergebn Physiol* , 1933 35, 982   This would apply to growing cells and to those which have ceased to grow (unpublished results) See also Jacques, A G , and Osterhout, W J V , *J Gen Physiol* , 1933–34 17, 727

[40] Bethe, A., *Arch ges Physiol* , 1916 163, 147

[41] Blinks, L R., *J Gen Physiol* , 1933–34, 17, 109

[42] Blinks, L R., *J Gen Physiol* 1932–33 16 147

W since they would tend to pass out by diffusion or to be used up in metabolism   (4) There may be a temporary increase in the mobility of K^+ or Na^+ due to changes in the composition of Y produced by the action current

When the current is interrupted before recovery is complete the process of restoring the positive potential of the resting state will continue because potassium[43] will be moved into the sap from W by the forces which normally produce this movement in the resting state of such cells as *Nitella* and *Valonia* [44]   This will be aided by the ingoing current which according to the local circuit theory occurs during recovery [45]

An objection to the idea that the action curve is due to the outward movement of cations is voiced by Gasser[38] on the ground that a subthreshold current which would carry ions outward fails to produce an action current   It seems to the writer that this might be due to two causes (1) potassium tends to move inward in the resting state[39] and the forces causing this must be overcome[46] before K^+ can travel outward, (2) there must be sufficient outgoing current to produce the necessary increase in permeability [47]

Blinks[33] suggests that the action current in *Nitella* may be accounted for by changes of hydrogen ion concentration   This view (which is in harmony with the ideas of Bethe[40]) is supported by very interesting experiments on *Halicystis* [41]   It seems possible that other chemi-

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[43] In this process potassium presumably moves chiefly in molecular form through Y Cf Osterhout, W J V , *Ergebn Physiol* , 1933, **35**, 994, 1003

[44] This will be aided by the return of the normal mobility of K^+ in Y and consequently of a higher outward diffusion potential

[45] This will depend somewhat on the position of the spot   If the action current proceeds from left to right a spot at the extreme right end of the cell will not receive much ingoing current of this sort

[46] An example of this is seen in certain models (*cf* Blinks, L R , *J Gen Physiol* , 1930–31, **14**, 127, also footnote 39)   Even in cells in which potassium no longer moves in the resting state but has reached an approximate equilibrium (as might happen in cells which were not growing) energy will be required to upset this equilibrium and cause K^+ to move outward

[47] This is evidently not produced by an ingoing current (perhaps because a substance must be carried from the sap into the protoplasm) nor by a current which increases very slowly

cal changes may be important   They may, for example, modify the protoplasmic surface and make it insensitive to potassium (as in experiments in which the surface is leached[48]), the changes produced in nerve by lack of oxygen[49] may be recalled in this connection

Further discussion of theories, particularly of those not dealing directly with *Nitella*, seems unnecessary   The hypothesis proposed in the present paper has some interest since it explains the form of the action curve in *Nitella* much more completely than other hypotheses (further evidence of this will appear in subsequent papers)   Its assumptions are in line with the local circuit theory which postulates an outward flow of current and consequently an outward movement of cations at the point of stimulation   the hypothesis here outlined ascribes chief importance to the movement of K^+ whose effect on *Nitella* is much greater than that of other cations

SUMMARY

The outstanding features of the action curve in *Nitella* are explained as due to the movement of potassium ions accompanied by increase of permeability   This may be useful as a working hypothesis since it accounts not only for the normal behavior but also for many striking deviations which will be treated in subsequent papers

The views here set forth are in harmony with the local circuit theory of stimulation

[48] Osterhout W J V , and Hill S E   *J Gen Physiol* , 1933–34, **17**, 87 99 105
[49] Hill A V , Chemical wave transmission in nerve   New York   The Macmillan Co , 1932   Cowan S L   *Proc Roy Soc London, Series B* 1934, **116**, 216

THE KINETICS OF PENETRATION

IX. MODELS OF MATURE CELLS

BY S. E. KAMERLING AND W. J. V. OSTERHOUT

(From the Laboratories of The Rockefeller Institute for Medical Research)

Models have been constructed[1] which in certain respects resemble living cells In such models potassium salts pass from an aqueous solution A through a non aqueous layer B into the artificial sap C where they react with CO_2 to form $KHCO_3$ Potassium reaches a much higher concentration in C than in A in consequence water enters C Eventually a steady state is reached in which water and electrolyte enter in a constant ratio and the volume of C increases while its composition remains approximately constant This seems to be analogous to what happens in actively growing cells

Can we also imitate mature cells in which growth has ceased? This involves some interesting problems In so far as the cessation of growth is due to a decrease in metabolism it would seem possible to imitate it in the model by decreasing the amount of CO_2 in the artificial sap (in C)

To test this we set up a model in which B consisted of 70 per cent of guaiacol plus 30 per cent p cresol (called G C mixture) By shaking G C mixture with 0 05 M KOH we obtained organic potassium salts called collectively KG this solution flowed through A (during the entire experiment), keeping its composition approximately constant [2] A and B were stirred mechanically

In previous experiments, where C consisted at the start of distilled

[1] Osterhout, W J V and Stanley, W M *J Gen Physiol*, 1931–32, 15, 667
Osterhout W J V, and Kamerling, S E, *J Gen Physiol*, 1933–34 17, 507
Osterhout, W J V Kamerling S E and Stanley W M, *J Gen Physiol*
1933–34, 17, 445, 469
[2] Some of the KG was converted to $KHCO_3$ see Osterhout W J, and
Stanley W M *J Gen Physiol* 1931–32 15, 667

water through which CO₂ was bubbled, it was found that the con-
centration of $KHCO_3$ in C increased to about 0 63 M   In the present
experiment 0 49 M $KHCO_3$ was placed in C at the start to save time
and the accumulation of potassium proceeded from that point   Pure
CO_2 was bubbled through C at the start   this procedure was changed
later as described in subsequent pages

Model I[1] was used (Experiment 129) B consisted of 1100 cc G C mixture
C contained at the start 68 cc   The CO_2 was passed through a solution of approxi-
mately the same osmotic pressure as that in C so as not to add or subtract much
water from C When it was desired to reduce the percentage of CO_2 nitrogen
was added to it and the bubbling was continued at the usual rate

The concentration of potassium in C rose steadily until it became
about 0 63 M where it remained approximately stationary although
the volume continued to increase   This is due to the fact that po-
tassium and water enter in a fixed ratio

The CO_2 supply was then decreased (at 480 hours, as shown in Fig
1) to about one-fourth [3] this caused the concentration of potassium
to fall off, but it subsequently rose again   Air was then substituted
for CO_2 and the concentration of potassium fell abruptly   This was
due in part to the entrance of water (as shown by the volume curve)
and in part to the exit of potassium (as shown by the curve of milli-
moles)   Potassium probably passed out[4] as KG, KOH, and $KHCO_3$

Beginning at 2282 hours, 1 7 per cent of CO_2 (by volume) was
bubbled through C The concentration of potassium began to in-
crease and the increase became more rapid when 5 per cent CO_2 was
used   On changing to 1 per cent (followed by 0 8 and 0 5 per cent
CO_2) the concentration of potassium fell off (but the volume continued
to increase)

These results show that the increase in volume of the artificial sap
depends on the supply of CO_2 and it seems probable that this is also
true of the living cell

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[3] This was done by mixing nitrogen with the CO_2 and bubbling at the same
rate as before

[4] Since the partition coefficient of $KHCO_2$ is very small as compared with that
of KG its concentration gradient in B and rate of movement in B is doubtless
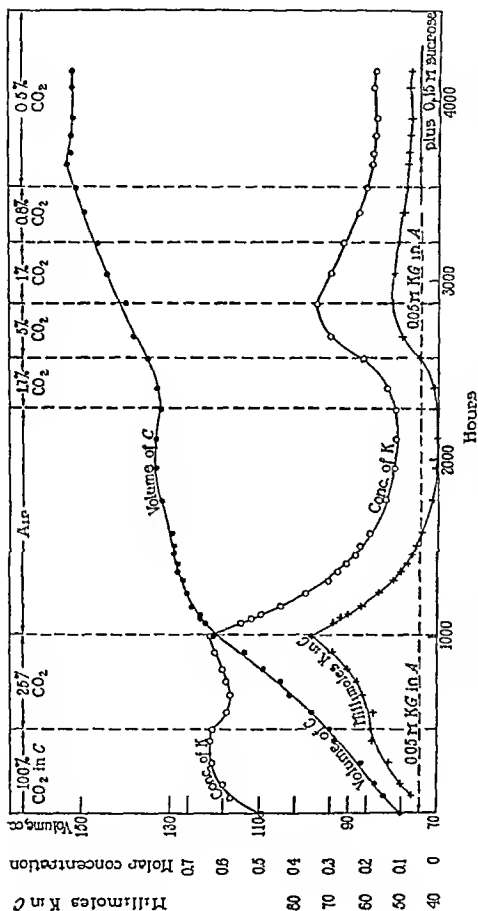correspondingly low

FIG 1 Shows the volume of the artificial sap and its content of potassium expressed as molar concentration or as total millimoles present At the start pure CO_2 was bubbled through C at 480 hours this was replaced by a mixture of 75 per cent nitrogen + 25 per cent CO (by volume) at 1010 hours this was replaced by air Subsequently CO was mixed with nitrogen to give the following percentages (by volume) at 2282 hours 1 7 per cent at 2566 hours 5 per cent at 2876 hours 0 8 per cent, at 3210 hours 0 5 per cent At 3646 hours enough sucrose was added to A to make its concentration 0 15 м

In order to imitate mature cells which have ceased to grow we must stop the increase in the volume of C   Three factors may be considered in this connection

1   Adjusting the concentration of electrolyte in C until the amount going out is equal to that going in   This can be controlled in part by varying the supply of CO_2 in C and the concentration of the penetrating electrolyte in A

2   Imposing a mechanical restraint such as is found in the cell wall of *Nitella* which checks the entrance of water and the increase in volume in spite of the relatively high osmotic pressure in the sap This cannot be done in the model

3   Placing in the external solution a substance which enters slowly and so helps to counterbalance the osmotic pressure of the artificial sap and retard the entrance of water, as happens with NaCl in the case of *Valonia*   We might employ an electrolyte in the model for this purpose   but it seemed more convenient to use sucrose   This was added in sufficient amount to make its concentration 0 15 M, thus making the osmotic pressure in A approach that in C (this imitates the condition found in *Valonia* where the osmotic pressure in the sap is only slightly higher than in the sea water)

As a result of this procedure the volume remained constant within 1 cc for about 2 weeks (the concentration of potassium was 0 18 M or 3 6 times that in the external solution)   The experiment was then discontinued

We may conclude that it is possible by means of a model to imitate certain important features of mature cells i e , to produce a steady state in which the volume and composition of the artificial sap remain approximately constant   the concentration of potassium being higher inside than outside   This is quite different from the steady state described in previous papers in which the composition of C remained constant while its volume increased (this steady state apparently resembles that in the growing cell)

DISCUSSION

The temporary nature of accumulation is clearly brought out by this experiment for as soon as we stop bubbling CO_2 in C the con-

⁵ Osterhout, W J V , and Kamerling, S E , J Gen Physiol , 1933–34, 17, 507

centration of potassium in C falls off Eventually A and C will be come identical since all the substances present can move through B

In previous experiments with models a steady state was achieved in which the composition of C remained constant while its volume increased This would correspond to the condition of an actively growing cell

In the present case both the composition and the volume of C were kept approximately constant in the final phase of the experiment The concentration of potassium was considerably higher than in A This would correspond to the condition of a cell which had ceased to grow

Under these conditions the entrance of potassium is equal to its exit The entrance of potassium will continue as long as the external activity product $[K] [G]$ is greater outside than inside The relation of this product in C to that in A depends chiefly on the following

1 On entering C, KG reacts with H_2CO_3 to form $KHCO_3$ and HG (guaiacol) Hence the more rapidly CO is bubbled through C the lower the product $[K] [G]$ and the greater the rate of entrance of potassium (since its concentration gradient in B will be larger[6])

2 The movement of $KHCO_3$ from C to A

3 The formation of $KHCO_3$ in C raises the osmotic pressure, causing water to enter and lowering the concentration of potassium This can be controlled by adding to A substances which enter slowly (or not at all) by which the entrance of water is checked Hence the concentration of potassium in C can be raised by adding such substances to A [7]

In the living cell the entrance of water can be hindered by mechanical restraint such as the cell wall of plant cells or by the pressure of surrounding tissues It can also be brought about by the presence in the external solution of a substance which penetrates slowly (or not at all), in *Valonia* the NaCl of the sea water acts in this way

In addition to these factors there are doubtless others, for example, in mature cells the protoplasm may become less permeable to certain

[6] Osterhout, W J V *J Gen Physiol* 1932–33 16, 529 Osterhout, W J V, Kamerling S E, and Stanley W M, *J Gen Physiol* 1933–34 17, 445

[7] This statement relates to substances which do not greatly lower the value of the product $[K][G]$ for example, it would not apply to the addition of HCl

electrolytes or to water   Certain erythrocytes which are said to be impermeable to potassium must have taken it up in their earlier stages of development

SUMMARY

To imitate cells which have ceased to grow we have made models in which artificial sap is separated from the external solution by a non-aqueous layer (representing the protoplasm)   A stream of CO_2 is bubbled through the artificial sap to imitate its production by the living cell

Potassium passes from the external solution through the non-aqueous layer into the artificial sap and there reacts with CO_2 to form $KHCO_3$ its rate of entrance depends on the supply of CO_2 Hence the increase of volume depends on the supply of CO_2 (as is probably true of the living cell)

By regulating the supply of CO_2 and the osmotic pressure we are able to keep the volume and composition of the artificial sap approximately constant while maintaining a higher concentration of potassium than in the external solution   In these respects the model resembles certain mature cells which have ceased to grow

THE ACCUMULATION OF ELECTROLYTES

VII Organic Electrolytes

Part 1

By A G JACQUES

(From the Laboratories of The Rockefeller Institute for Medical Research)

It has been suggested that potassium may enter *Valonia* chiefly as KOH which reacts with an organic acid HA in the sap to form KA, after which A is exchanged for chloride so that potassium accumulates as KCl   This is not contradicted by any known facts and it is sup ported by a variety of evidence, inasmuch as this has recently been discussed[1] it need not be cited here   Other cations may act similarly in this and in other cells

This means that the entrance of cations is accompanied by the formation of organic salts whose presence could be demonstrated if they existed in sufficient quantity   For this reason it seemed de sirable to examine the sap of certain plants in which considerable amounts of organic acid could be expected

The plants studied were *Rheum rhaponticum*, L (the rhubarb used as a vegetable), *Rumex acetosella*, L (field or sheep sorrel), and *Oxalis corniculata*, L (wood sorrel) mixed with a small amount of *Oxalis filipes*, Small

A complete analysis of the plant sap for all ions, including the or ganic anions, is unfortunately not possible at the present time since to deal quantitatively with complex mixtures of organic anions a very large amount[2] of material must be available   But the analyses here given throw some light on the question at issue

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[1] Osterhout, W J V, *Ergebn Physiol*, 1933 35, 967   Jacques A. G, and Osterhout, W J V, *J Gen Physiol*, 1933–34 17, 727

[2] For reasons which will be discussed in Part 2 we confined our investigation to leaves these were available only in limited amounts

Experimental Procedure

After collection the plants were kept in a moist cool place   Only the thinner parts of the leaves were used for the extraction of sap   the leaf petioles were rejected, and in the case of *Rheum* the thick midvein of the leaf also   Just before use the leaves were rinsed first in tap and then in distilled water and were dried between sheets of filter paper   They were then killed by dipping for a few seconds in ether according to the method of Chibnall,[3] and, after the removal of excess ether by evaporation, transferred to a screw press (tincture press) in which the sap was expressed   In all cases moderately cloudy saps, orange to reddish in color and nearly free of chlorophyll, were obtained   Immediately after expression the sap was treated with an equal quantity of alcohol slightly acidified with acetic acid (or in two cases strongly acidified by the solution of dry HCl gas)   A small portion of untreated sap was retained in each case for the determination of acidity, pH, and, where HCl-alcohol was used, for chloride ion

In the treated saps after 24 hours in the ice box a heavy grey-white precipitate had formed   This was removed by centrifugalizing at 4000 R P M. for 20 to 30 minutes   The decanted sap was perfectly clear, but still highly colored   Microscopically the precipitate was found to consist of a small amount of fibrous material, a great deal of poorly crystallized material, probably organic, and, except where the strongly acidified alcohol was used, a few well defined crystals   These might have been calcium or magnesium oxalate or phosphate   Analyses were then made according to the following procedures

Phosphate—This was determined by the ammonium phosphomolybdate method of Woy,[4] the precipitate being subsequently purified according to the recommendations of Treadwell[5]   The sample was first dried on the water bath to remove alcohol, then treated several times with concentrated nitric acid to destroy organic matter   After removal of the excess acid by evaporation just to dryness, the residue was taken up in water, and the precipitation was carried out in the prescribed manner

Calcium and Magnesium—Calcium and magnesium were found to be present in the two cases where the sap was treated with the HCl-alcohol mixture

Preliminary experiments indicated that when the sap was made alkaline with ammonia a flocculent precipitate of organic matter was thrown out   Accordingly it was considered advisable to get rid of organic matter first   The sap was dried on the water bath, and in the oven at 130°, and finally ignited at low red heat until a fairly white ash was secured   The ash was extracted with hot dilute HCl to ensure the solution of oxychlorides and of $Mg_2P_2O_7$ After filtration the

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[3] Chibnall, A C , *J Biol Chem* , 1923, **55**, 333

[4] Woy, R , *Chem -Ztg* , 1897, **21**, 442

[5] Treadwell, F P , and Hall, W T , Analytical chemistry   II Quantitative analysis, New York, John Wiley and Son, 6th edition, 1924, 383

calcium was precipitated in the filtrate by means of ammonium oxalate in the presence of ammonia and NH_4Cl A visible precipitate was produced but after ignition the amount of CaO was too little to weigh on an ordinary balance   Magnesium was precipitated just below red heat in the filtrate from the calcium determination as $MgNH_4PO_4$ and ignited to $Mg_2P_2O_7$, according to the method of Schmitz [6]

Sodium and Potassium —These were determined together as the sulfates, and potassium as potassium chlorplatinate according to the Lindo Gladding procedure [7] the sodium being then calculated by difference   The sample was dried and ignited just below red heat   In order to avoid the loss of KCl ignition was carried only far enough to decompose the organic compounds   The black residue was broken up and extracted with hot water   Where phosphate was present it was removed by the familiar basic ferric acetate method and magnesium (in the two cases where it was present) was removed by means of the Schaffgottsche reagent [8]   The filtrate from these separations containing sodium, potassium and ammonium sulfate and chloride was evaporated to dryness, and the residue ignited below red heat to remove ammonium   It was then taken up in water treated with a few drops of concentrated sulfuric acid   The solution was then evaporated first on the water bath and then on the sand bath and finally ignited at low red heat to decompose the acid sulfates   After weighing the salts were dissolved and the potassium was determined in the solution as described above

Sulfate —Sulfate was determined in the alcohol mixture directly by precipitation as barium sulfate   The precipitate was filtered through paper and ignited to get rid of occluded organic matter

Chloride —In order to remove organic materials which might reduce silver nitrate the sample was ignited below red heat until all the organic matter was decomposed   The black residue was extracted, filtered, and the chloride was determined gravimetrically in the filtrate as silver chloride

Acidity —The acidity of the sap was determined by titrating with CO_2 free sodium hydroxide to the change point of phenolphthalein

The pH was determined in two cases by the glass electrode [9]

Nitrate —Tests were made for nitrate by means of nitron and diphenylamine but only doubtful traces were found

Organic Acids —Unfortunately the literature contains no satisfactory procedures for the determination of the mixtures of organic anions in small quantities of material   Vickery and Pucher[10] have devised methods for the anions

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[6] Schmitz, B   *Z anal Chem* 1906 45, 512

[7] See Treadwell F P and Hall W T, Analytical chemistry   II Quantitative analysis, New York John Wiley and Son 6th edition 1924, 79

[8] See Treadwell F P and Hall, W T, Analytical chemistry   II Quantitative analysis, New York, John Wiley and Son, 6th edition 1924, 62

[9] These determinations were kindly made by S E Hill

[10] Vickery, H B and Pucher G W   *Bull Conn Agric Exp Station, No 323,* 1931

present in tobacco leaves   These methods depend on precipitating the anions as barium salts, decomposing the salts by sulfuric acid, extracting them into ether, and esterifying, and fractionally distilling the esters, and are better adapted to large samples

In view of these facts it was deemed inadvisable to attempt at this time any thorough investigation of the acids present in our plants   In all cases oxalic acid was identified by means of the fairly characteristic calcium oxalate crystals [11]

<div align="center">RESULTS</div>

The results of the analyses are given in Table I

Evidently the leaf saps were mixtures derived from three possible sources, the vacuoles, the protoplasm, and the intercellular spaces However, we are chiefly interested in the ionogenic constituents of the first

Chibnall[3] has shown that the method of extracting the leaf sap by treating the leaves with ether and then pressing results in the expulsion of the vacuole sap but not of the protoplasm, the cells being flattened but not apparently ruptured [12]

The admixture of the intercellular fluid with sap from the vacuoles cannot be avoided but it does not seem important in this work since it would not be expected to increase the ratio of cations to anions, which is the essential point involved in the present investigation   The intercellular fluid probably consists almost entirely of inorganic compounds, being mostly the external solution (somewhat concentrated by evaporation) plus CO_2 and perhaps some other materials given out by the cells   Its content of solutes is very small as compared with the sap[13] and the amount mixed with the sap cannot be large since the intercellular spaces are mostly filled with gases

In the discussion which follows it will be assumed that the substances reported were present in the clarified saps as ions or parts of dissolved ionogenic substances   This assumption, which we believe to be valid, will be discussed more fully in Part 2

[11] Behrens, H, and Kley, C, Organische mikrochemische Analyse, Leipsic, Leopold Voss, 2nd edition, 1922, 332

[12] In the experiments here described the saps were chlorophyll-free, which indicates that they did not contain much protoplasm

[13] In this connection see the work of Shedd, O M, and Kastle, J H, *J Am Chem Soc*, 1912, 34, 1415, on the liquid exuding from cut branches of the grape

TABLE I

Analyses of Plant Leaf Saps

Plant	Equivalent concentrations									Cations/Anions	Acidity	pH
	K+	Na	Ca++	Mg++	H2PO-	SO -	Cl	Total cations	Total anions			
Rheum rhaponticum, L	0 0702	0 0130	None	None	0 0038	Trace	0 0703	0 0832	0 0241	3 5	0 0471	
	0 0722	0 0104	None	None	0 0024	0 0022	0 0187	0 0826	0 0233	3 5	0 0775	4 49
	0 0871	0 0046	Trace	0 0433	0 0039	0 0020	0 0199	0 1350	0 0258	5 3	0 0577	
Rumex acetosella L	0 0330	0 0091	None	None	Trace	0 0018	0 0066	0 0421	0 0084	5 0	0 0975	
	0 0174	0 0046	Trace	0 0101	0 0063	0 0045	0 0074	0 0321	0 0182	1 8	0 0988	3 32
Oxalis (90 per cent *Oxalis cornic ulata* L + 10 per cent *Oxalis filipes*, Small)	0 0735	0 0060	None	None	0 0057	0 0032	0 0117	0 0795	0 0206	3 9	0 1007	1 2†

* Determined by titration to the change point of phenolphthalein
† Smith, E F , and Quirk A J *Phytopathology*, 1926, 16, 491

In Table I the equivalent concentration of the phosphate ion has been calculated on the assumption that in the saps we have studied phosphoric acid is monobasic   The pH of rhubarb sap is about 4 5 (Table I),[14] and pK₂ for phosphoric acid is 6 9, so that according to the Henderson-Hasselbalch equation only about 0 4 per cent of the total phosphate can be present as HPO_4^{--} Naturally in the saps of *Rumex* and *Oxalis*, which have still greater hydrogen ion activities, the proportion of bivalent phosphate will be less   It may be pointed out that even if the phosphate ion is assumed to be trivalent it does not alter the major conclusion which may be drawn from the analyses

The data show that in these saps the cation equivalents exceed the anion equivalents, the smallest ratio of cation to anion being 1 8 and the largest 5 3   The average is 3 8 [15]   The excess of cations is, of course, paired with various organic anions

As to the nature of these anions, it has already been stated that the quantitative separation of the complicated mixture of organic anions derived from plant sources has not yet been accomplished satisfactorily and no attempt has been made to solve this problem in the present case

In *Rheum rhaponticum*, L , according to Wehmer,[16] benzoic and gallic acids have been detected, and in a number of closely related species, oxalic and malic acids   There is no doubt that the last two are the most important   According to Bau[17] from 0 05 to 0 5 per cent of oxalic acid exists in fresh young leaves of rhubarb   Maue[18] reports 0 358 per cent oxalic acid in fresh rhubarb leaves and Tsakalotos[19] 0 46 per cent in fresh leaves of *Rheum undulatum*   In *Rumex* and *Oxalis* oxalic acid is said to be abundant[20] and this is confirmed by our qualitative tests

Probably there are small amounts of proteins present in all saps, but at the low pH's encountered in the plants studied by us it is probable that they were acting as cations   In this connection it is interesting that Chibnall and Grover[14]

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[14] Chibnall, A C , and Grover, C E , *Biochem J* , London, 1926, 20, 108

[15] If phosphate is taken as bivalent the ratio of cations to anions in the second sample of *Rumex* is still 1 30

[16] Wehmer, C , in Klein, A , Handbuch der Pflanzenanalyse, Vienna, Julius Springer, 1932, 496

[17] Bau, A , *Z tech Biol* , 1921, 8, 151

[18] Maue, G , *Z Nahr -Genussm* , 1920, 40, 345

[19] Tsakalotos, A E , *Schweiz Apoth -Ztg* , 1919, 57, 303

[20] Czapek, F , Biochemie der Pflanzen, Jena, Gustav Fischer, 3rd edition, 1922–25, 69

found that no soluble cytoplasmic protein could be extracted from *Rumex* and *Rheum* because the sap was below the isoelectric points of the proteins

It will be observed that calcium and magnesium were present in but two samples of sap This is probably due to the preliminary treatment of the sap after expression With the exception of the two samples mentioned the sap was treated at once with an equal volume of alcohol and a few drops of glacial acetic acid, and chilled for about 24 hours Under these circumstances there separated out not only a heavy organic precipitate, but also crystalline material which contained traces of calcium and much magnesium and which may have been either calcium and magnesium oxalate or other organic salts or phosphates or all of these Assuming that these substances were originally retained in solution in the sap due to its low pH their deposition before analysis does not vitiate our conclusion as to the excess of cations In the exceptional cases the salts probably did not deposit because of the very low pH due to the added HCl

The small amount of calcium in the sap recalls the situation in *Valonia* [21] Recent experiments on *Nitella* support the suggestion[22] that the chief function of calcium may be to prevent certain organic substances from leaching out of the cell surface and in that case calcium would not be necessary in the vacuole since it contains these organic substances

These results are in harmony with those of Teakle[23] who finds an excess of inorganic cations over anions in the sap expressed from wheat plants

It is clear that the excess of cations over anions can be explained on the assumption that the cations have entered as hydrates This suggestion has already been made on other grounds[24] and has been applied to models [25]

[21] Osterhout, W J V *J Gen Physiol* 1922–23, 5, 225 Here the figure for calcium in parts per thousand is 10 times larger than it should be The method is such that the molar concentration given may be too large

[22] Osterhout, W J V *Ergebn Physiol*, 1933 35, 967 Osterhout, W J V, and Hill, S E, *J Gen Physiol*, 1933–34, 17, 87

[23] Teakle, L J H, *Plant Physiol*, 1929, 4, 211

[24] Osterhout, W J V, *Science*, 1912, 36, 571 *Proc Soc Exp Biol and Med*, 1926–27, 24, 234 *Bull Nat Research Council, No 69* 1929, 170 *J Gen Physiol*, 1930–31 14, 285, *Biol Rev* 1931, 6, 369 *Ergebn Physiol* 1933 35, 967 Jacques, A G and Osterhout, W J V *J Gen Physiol*, 1933–34, 17, 727

[25] Osterhout, W J V and Stanley W M *J Gen Physiol*, 1931–32, 15, 667 Osterhout W J V *J Gen Physiol* 1932–33 16, 529 Osterhout W J V, and Kamerling S E, *J Gen Physiol* 1933–34, 17, 507 Osterhout W J V Kamerling S E and Stanley, W M *J Gen Physiol*, 1933–34 17, 445, 469

It is, of course, possible that cations enter in exchange for H+ produced in the cell but it seems improbable that this plays an important rôle [26]   There are, however, other possibilities which will be discussed in the second part of this paper

SUMMARY

The inorganic constituents of the sap of *Rheum* (rhubarb), *Rumex* (field sorrel), and *Oxalis* (wood sorrel) show a great preponderance of cations over anions, as would be expected if the cations entered chiefly as hydrates (other possibilities will be discussed in Part 2)

[26] Osterhout, W J V , *Ergebn Physiol* , 1933, **35**, 994

RESULTS OF IRRADIATING SACCHAROMYCES WITH MONOCHROMATIC ULTRA-VIOLET LIGHT

II THE INFLUENCE OF MODIFYING FACTORS

BY ROBERT H OSTER

(From the Laboratory of General Physiology, Harvard University, Cambridge)

It bas been shown previously (1, 2) that the action of monochro matic ultra violet radiation on the yeast *Saccharomyces cerevisiae* is not an all or none effect, but is a graded result varying from induction of simple inability to form normal sized colonies to "death" of the cell, through different degrees of damage, and involving in some stages the formation of giant cells The mean "survival" curves for irradi ated 24 hour cultures (2) resemble the curve for a first order process, but on this assumption divergences indicate the presence of modify ing factors

The present paper deals with several factors which may modify the absorption of energy and the cell processes resulting from irradia tion of yeast with ultra violet light

Age of the Cells

Similar to the effects observed with other organisms (3, 4), the age of the yeast culture has a marked influence on the relative resistance of the cells to lethal irradiation, as measured by the energy required to suppress budding (2) This is illustrated by a comparison of the results obtained on irradiating two cultures of widely different periods of incubation, (A) a 24 hour culture and (B) a 15 day culture of yeast, both incubated at 25°C and irradiated in a large quartz mono chromator at the wave length 2535 Å u as previously described (2) after inoculation on malt agar contained in small Petri plates As shown in Fig 1, from 20 to 50 per cent more incident energy to pro duce a given effect is required for the 15 day culture than for the 24 hour cultures This is best explained on the basis that the greater

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resistance of the 15 day culture is due to the greater proportion of cells in the resting stage

The integral curve of reproduction of yeast, under the conditions observed in these experiments (2), shows that in a 24 hour culture, cell division is still going on at a fairly rapid rate so that the cells of a given inoculated plate will be in various phases of the reproductive cycle at the time of irradiation   The results obtained by Wyckoff and Luyet (1) using 15 day cultures of yeast, in which they found the
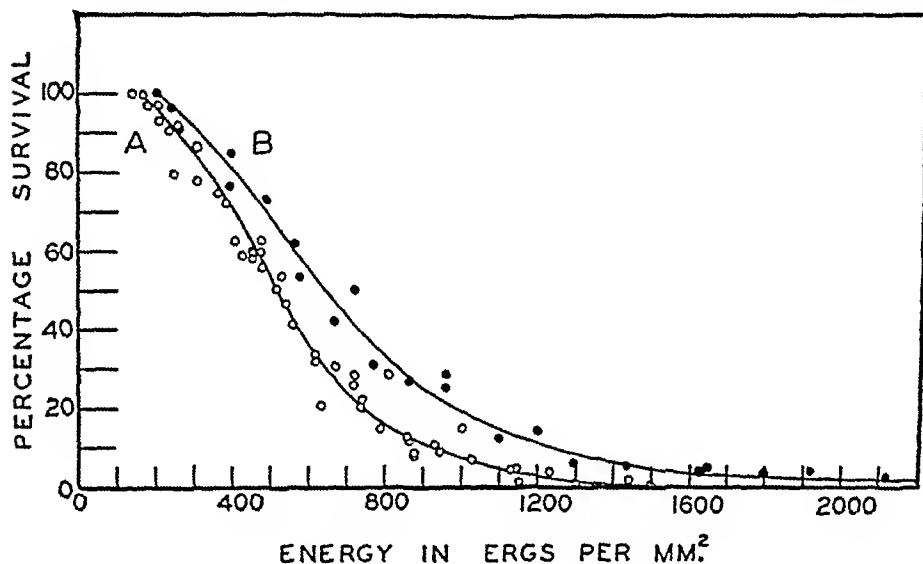
FIG 1   Curves showing the difference in the rate of killing as a function of the incident energy, for yeast cells exposed to monochromatic ultra-violet radiation (2535 Å u)   Curve A, 24 hour culture   Curve B, 15 day culture   The plotted points give the percentages of cells forming two or more daughter cells, based on counts of 100 to 300 cells (cf Oster, 1934–35)

survival curve followed a multiple hit to kill relation, indicate that the reproductive state of the cell is an important factor in its resistance to changes produced by ultra-violet irradiation

In contrast to the results obtained with ultra-violet irradiation, yeast cells exposed to X-radiation (1, 5) show a large number of two-cell groups even after long exposures   Lacassagne and Holweck (5) have referred to this ability of the cell to form one bud and no more as a case of "deferred death"   They also state that cells undergoing rapid division were more resistant than the older resting cells   These

results, along with those of Strangeways and Hopwood (6), that tissue cells in the phase just preceding the prophase are especially susceptible to X-radiation, suggest that ultra-violet energy is absorbed by the nucleus with a slight but significant difference from the way in which X rays, cathode rays, and alpha particles are absorbed The reasons for this difference have recently been treated quantitatively by Holweck (7)
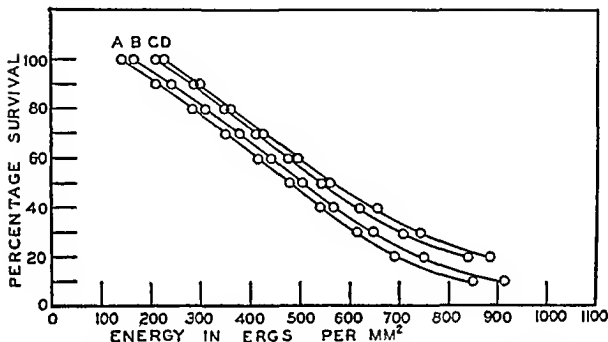
Fig 2 Survival curves of yeast cells exposed to monochromatic ultra violet radiation of wave length 2535 Å u at four different temperatures A at 29 5°C B at 24 0°C , C at 16 0°C D at 8 0 C The points indicated by clear circles are mean values at 10 per cent intervals obtained from the smoothed curves (cf Fig 1) secured in several experiments at each temperature

Temperature in Relation to the Lethal Effect

All of the data given in previous papers (2) dealing with the inhibitory and lethal effects of ultra violet energy on yeast were obtained on cells exposed at the temperature of the laboratory, between 22° and 25°C Within this range no evident effects on the inhibitory process could be observed, indicating a low temperature coefficient

Determination of the temperature coefficient of the lethal action was made from data obtained by a series of observations on cells irradiated at 2535 Å u at four temperatures (8°, 16°, 24°, and 29 5°C)

The tests were performed as before (2), but with the inoculated agar plate and glass slide exposed in a water-jacketed brass cell with the aperture on the exposed side covered by a crystal quartz plate and that on the back with a glass plate for locating the agar plate in the path of the light beam   Three tubes provided for the water inlet and outlet and for the insertion of an accurate thermometer into the water chamber   With a steady stream of water from a storage vessel it was found that the temperature could be controlled to within plus or minus one-half of one degree for the duration of a given series of exposures   Before each plate was exposed it was allowed to remain in the chamber 10 minutes in order to come to the observed temperature   The mean values for several experiments at

TABLE I

Data Used in the Determination of the Temperature Coefficient for the Lethal Action of Ultra-Violet Radiation of Wave-Length 2535 Å u on Yeast

Killed	Energy required				Reciprocal of energy ratio for 10°C change		
	At 24°C	At 8°C		At 29 5°C	8–24°C		8–29 5°C
	Mean	Mean	Exp 91	Mean	Mean	Exp 91	Mean
	ergs per mm²	ergs per mm²	ergs per mm²	ergs per mm³			
20	311	360	345	285	1 10	1 07	1 12
30	378	425	412	350	1 08	1 05	1 10
40	440	495	478	416	1 07	1 08	1 09
50	503	560	542	480	1 07	1 05	1 08
60	566	653	615	542	1 08	1 05	1 09
70	648	745	695	615	1 08	1 05	1 09
80	751	885	800	690	1 10	1 04	1 13
					1 08	1 06	1 10

each temperature are plotted in Fig 2   The temperature coefficient was obtained by taking the reciprocal of the energy ratio for a 10° change (Table I)

Using the outer limits (29 5° and 8°C) an average value of 1 10 is obtained   Using the range between 8° and 24°, mean values for the 8° series give an average value of 1 08   When Experiment 91 is used, at 8°, in which the temperature could not be observed to change over the entire course of the exposures, a lower value of 1 058 is obtained for the range 8–24°   This is in good agreement with the low value 1 06 obtained for lethal action on bacteria (8)

The higher value (1 10) obtained when the 29 5° series is used may

be due in part to the influence of another reaction, since it has been demonstrated that 30°C is a critical temperature for this strain of yeast (9) The values obtained suggest that the effects produced by the radiation are physical or direct rather than chemical in nature

If the rate of "killing" is plotted as the logarithm of the energy required to "kill" a given percentage (50 per cent) against the recip-
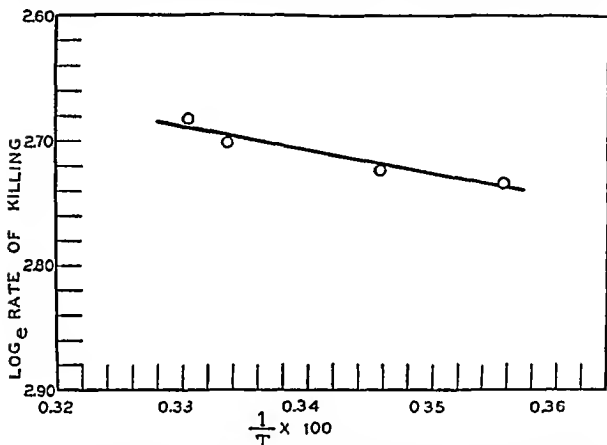
FIG 3 Graph showing the relation between the lethal action of ultra violet radiation at wave length 2535 Å u and temperature The points are plotted as the energy required to kill 50 per cent of the cells as a function of the reciprocal of the absolute temperature at 29 5° 24 , 16° and 8°C The temperature characteristic calculated according to the Arrhenius equation has the value 640

rocal of the absolute temperature, according to the equation of Arrhenius, the temperature characteristic can be determined for the process (Fig 3) This is found to have a mean value of about 640, a value much lower than any found for ordinary chemical processes (10)

Effects of Ultra Violet Energy upon the Nutrient Medium

To determine whether the irradiation of the malt agar medium was a factor in the inhibitory and lethal effects (11), plates of the medium

were exposed for periods ranging from 5 minutes to 2 hours, the yeast was then seeded on these surfaces by a fine mist from a vaporizer, the cells being allowed to settle on the agar with as little excess moisture as possible   No effect on the subsequent growth of the cells could be detected   As the droplets of water containing the yeast were so small as to show no visible moistening of the agar surface, it is unlikely that any toxic substances formed by the action of the radiation upon the medium could have been diluted to an appreciable extent

Test of Toxicity

To test the possibility that toxic substances formed in the cells killed by the irradiation may diffuse out subsequently and aid in the formation of the abnormal and inhibited cell groups observed, the following tests were made

Four malt agar plates were inoculated with a yeast suspension of standard turbidity and allowed to drain   In the meantime a heavy suspension of yeast from the same culture, filling a large Petri plate to a depth of 8 mm , was exposed at a distance of 20 cm from a horizontal quartz mercury vapor arc operated at 67 volts and 5 5 amperes During irradiation the suspension was stirred at frequent intervals and samples were removed at the end of 1, 5, 10, and 60 minutes exposure   Small drops of these samples were immediately pipetted on to marked areas of the plates previously seeded with non-irradiated cells   After 36 hours incubation at 25°C the plates were examined and the following observations made, (a) all of the areas covered by drops of the exposed suspensions showed the same number of normal colonies as the adjacent control areas, with the exception of the 1 minute exposure   The latter areas showed approximately three times the normal number of colonies, with 3 per cent of the colonies showing some giant cells   (b) In the 5 minute areas the normal sized colonies were surrounded by numerous small colonies and one- and two-cell stages   These small colonies are evidently survivors from the irradiated suspension   (c) In the 10 minute areas the normal sized colonies were surrounded by many one- to three-cell stages, many of which contained giant cells   (d) The 60 minute areas contained hundreds of single cells surrounding the normal colonies or

clumped at the edge of the area   The mean diameter of the normal colonies in the various areas was the same as that of colonies in the control regions

From these observations we may conclude that cells killed or damaged by ultra violet radiation, under the conditions here described, do not liberate toxic substances capable of affecting the subsequent growth of adjacent normal yeast cells

The Bunsen Roscoe Reciprocity Law

No extended series of experiments was made to test the validity of the Bunsen Roscoe reciprocity law   However, it was necessary to test the effects produced by the irradiation for variations of intensity of the same or somewhat greater degree than those commonly present in the actual experiments   In two different experiments the intensity of the incident light at 2804 Å u was decreased by 30 per cent by increasing the distance of the test object from the exit slit   This difference in intensity is considerably greater than any actually experienced at any given wave length during the course of the investigation (2)

On plotting the percentage survival against the energy incident upon the receiving surface for the two experiments (at 10 ergs per mm² per sec and 7 ergs per mm² per sec) it was found that the deviation between the two curves was considerably less than the maximum deviation between curves plotted from data obtained at constant intensity   During the course of the complete tests the actual variation of intensity of the incident light was never found to exceed 20 per cent, over all wave lengths, so that the Bunsen Roscoe law has been considered to be valid within the narrow limits of intensity variation permitted   For wide variations in intensity, however, it is probable that the law would not hold (8) and that the Schwartzchild exponent q in the equation $I^q t = A$, would be greater than one

CONCLUSION

Possible variation in the probability that absorbed quanta of ultra violet energy will produce observable inhibitory and lethal effects in the yeast cell, due to non uniformity in sensitivity of the different regions of the cell, may be further modified by the reproductive stage

of the cell at the time of irradiation   Tests of the survival of yeast cells of 15 day and 24 hour cultures indicate that the older resting cells are more resistant to ultra-violet irradiation effects than cells undergoing rapid cell division

The effects of temperature changes within the range of normal growth are evidently small as judged from the temperature coefficient (1 10) Possible inhibitory effects due to the action of ultra-violet radiation on the malt agar medium and to toxic substances diffused from cells killed by irradiation were not found under the conditions of the experiments

Tests of the validity of the Bunsen-Roscoe reciprocity law for variation in the intensity of the incident ultra-violet radiation up to 30 per cent indicate that for this range the rate of absorption of quanta by the cell does not produce any marked change in the lethal effects observed

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RESULTS OF IRRADIATING SACCHAROMYCES WITH MONOCHROMATIC ULTRA-VIOLET LIGHT

III The Absorption of Ultra Violet Energy by Yeast

By ROBERT H. OSTER

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It has been shown previously (1–3) that monochromatic ultra violet radiation produces inhibitory and lethal effects in the yeast *Saccharomyces cerevisiae* which are approximately the same at each wave length tested but require quite different incident energies to effect the same degree of reaction at different regions of the ultra violet spectrum (2)

The present paper deals with these differing energy requirements in the production of a given reaction end point over the region of the spectrum studied, as an index of the biological or lethal spectrum of yeast

The effectiveness of ultra violet energy in producing a given effect, whether it be inhibition of normal colony formation, cessation of metabolic processes such as respiration or fermentation, or the stoppage of growth and cell division, must be intimately related to its absorption by the yeast cell or by the medium upon which the cell grows No demonstrable effect of irradiating the malt agar medium could be observed Plates of the medium irradiated for 1 and 2 hours supported normal colony formation of yeast seeded immediately after the exposure Under the conditions of the tests the effect discussed must be directly upon the yeast cell

Measurement of the absorption of ultra violet energy by intact yeast cells was attempted[1] by the method used by Gates (4) in obtain

ing absorption curves for *Staphylococcus aureus* and *Bacillus coli*   A loopful of moist yeast cells was pressed between two crystal quartz plates and the thickness of the layer was measured by interferometry (4, 5)   The specimen was then set up in one optical path of a sector photometer, with a film of glycerol of practically the same thickness between similar quartz plates in the other path as a control

Two beams of light from the tungsten-iron spark source passed through the specimen and the control and were spread by a large quartz spectrograph into parallel spectra in the plane of a photographic plate   The light passing through the control could be varied by means of the sector vane to give a graded series of progressively lower intensities on the spectrogram against which the specimen spectra could be matched in the different wave-lengths

However, it was found that so great was the difference between the energy transmitted in the two paths that no points of equal blackening could be found on the plates within the range of the sector shutter   A pair of micro-Baly tubes were next used in place of the films, one tube containing an aqueous suspension of yeast cells of moderate density and the other a control suspension of an inert powder (India ink)   With this arrangement the length of the control suspension could be varied in conjunction with the sector shutter, or alone, over a wide range of transmitted energy   Points of equal blackening on the spectrogram were obtained by this method, but so great is the scattering and refraction by the chitinous walls of the yeast cells that no significant differences in the relative blackening could be discerned, i e , when a match at one wave-length (e g , 3100 Å u) is obtained all wave-lengths match over the range studied

Other methods were tried with similar results   Transmission of energy down to 2200 Å u indicates that it is probably refracted from cell wall to cell wall and thus masks any characteristic absorption pattern of the cytoplasm

However, an approximation of the absorption spectrum of the substance or entity within the cell which is so modified by the absorbed energy that injurious effects to the yeast cell result, is obtained by plotting the incident energies required to cause "death" of 50 per cent of the cells (*cf* Oster (2)) against the wave-length as shown in Fig 1, Curve A   Curve A indicates the different energy levels at

the wave lengths tested and shows a minimum energy requirement between 2600 and 2700 Å u , with another minimum indicated below 2300 Å u   Significant effects were not found above 3022 Å u , and at wave lengths below 2225 Å u the intensity was found to be so low as to be unsuitable for quantitative tests
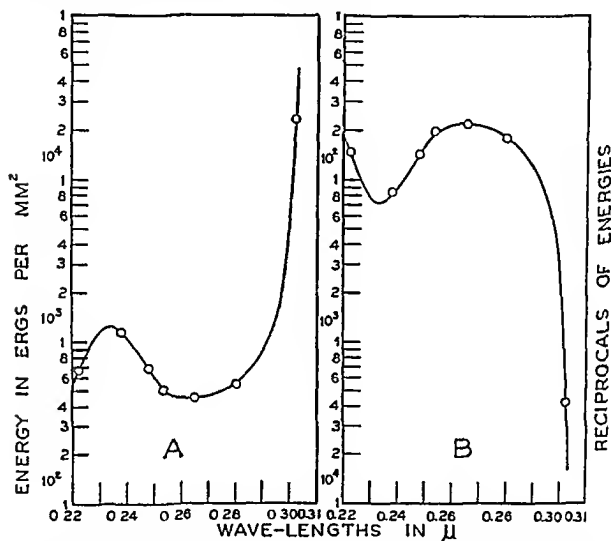

Fig 1 Comparison of the destructive efficiency of ultra violet energy on yeast at different wave lengths. Curve A incident energies involved in the ' killing of 50 per cent of *S cerevisiae* at different wave lengths   Curve B, reciprocals of the energies required to "kill" 50 per cent of the yeast cells

If we assume that the injurious effects in the yeast cells result from the absorption of light quanta, the significance of Curve A lies in the specific absorption of the different quanta by the cells, and the absorption curve (lethal spectrum) of the substance affected is more conventionally represented by plotting the reciprocals of the energies

(Curve B, Fig 1)   If the limitations are recognized, such an assumption is of value in the analysis of biological reactions in relation to absorbed radiant energy (cf Warburg (6), Gates (4, 8), Kubowitz and Haas (7))

A comparison of the energies required to suppress budding of 50 per cent of the cells irradiated shows that they range from 457 ergs per mm ^{2} at 2652 Å u to 23,500 ergs per mm ^{2} at 3022 Å u, or roughly a ratio of five times the energy range for *S aureus* (4)   The 50 per cent point was chosen because it falls at the most accurately determined part of the curve and because it represents a median index of the inhibitory process in the irradiated cells

DISCUSSION

Curve B of Fig 1 is essentially similar to absorption curves of certain enzymes (7, 9) and nucleoprotein derivatives (8)   In the location of its energy peaks on the wave-length scale the lethal spectrum of yeast thus obtained resembles the absorption curve of the pyrimidine bases of nucleic acid, cytosine and uracil (8)   These nucleoprotein derivatives are known to occur in yeast (10), and Jones and Perkins (11) have shown that cytosine along with guanine and adenine nucleotides can easily be isolated in quantity from yeast nucleic acid

The suggestion is made that the effects of ultra-violet irradiation may result from the absorption of energy by these nucleoproteins

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ADAPTATION OF CUTANEOUS TACTILE RECEPTORS   II

By HUDSON HOAGLAND

(From the Physiological Laboratory, Clark University, Worcester)

I

A method of recording action potentials from individual nerve fibers supplying single receptors in the skin of the frog has been described (Adrian, Cattell, and Hoagland, 1931, Hoagland, 1932) Leads are placed on a dorsal cutaneous nerve which has been cut near its entrance to the skin, and antidromic impulses are recorded   These impulses are set up by stimulating a single receptor supplied by an axon which branches in the dorsal root ganglion and sends a fiber out along the dorsal cutaneous nerve under investigation   Thus the impulses travel from the receptor to the ganglion and then out again to where they are recorded without having entered the central nervous system or passed a synapse   The action potentials are amplified and recorded with a Matthews oscillograph used in conjunction with a camera, a standing wave screen, and a loud speaker   Stimulation of the tactile receptor preparation at any desired frequency, duration, or intensity may be secured by applying to the skin surface a jet of compressed air interrupted by a toothed disc fitted to a flexible shaft and revolved by a motor

"Adaptation" to a constant pressure was found to be very rapid—only one or two impulses being set up   With repeated air blasts of short duration (5σ) only a single impulse is set up for each puff of air   If the frequency is sufficiently great the end-organ soon fails to follow every stimulus, more and more impulses being dropped out until the response ceases entirely   The failure depends not only upon the frequency of stimulation but upon the duration of the stimulus and the interval between stimuli   The time of complete failure of response may be regulated in terms of these variables from a few seconds to more than an hour (Cattell and Hoagland, 1931)

Studies have been made of the rate of adaptation of tactile receptors as a function of the properties of a series of discs used to interrupt the air jet (Hoagland, 1932–33 a)   Empirical equations were determined for the failure of response, and equations relating the velocity constants of these equations to the ratio of $S/R = \dfrac{\text{Stimulating time}}{\text{Time between stimuli}}$ as determined by the relative area occupied by the notches and the intervening solid material of the discs

<div align="center">II</div>

A general hypothesis concerning a possible mechanism of adaptation, that is, of failure of nerve impulses set up in response to repeated pressure stimulations of single tactile receptors, is here presented and discussed in the light of a group of experimentally ascertained facts concerning the phenomenon

Encapsulated receptors in frog's skin are histologically rare   The receptors ($\pm$ 200 per c̄m ^{2}) may be regarded as free nerve endings branching among cells of the epithelium (cf Adrian, Cattell, and Hoagland, 1931)   They may be viewed as stimulated directly by mechanical movement of the skin which sets up tension and stretches the filamentous endings   The stimulus may thus be regarded as acting in a way similar to that of pressure on an excised nerve trunk— a constant applied pressure, in general, sets up impulses only momentarily during its application and again occasionally on its removal, the nerve apparently "adapting" itself to the constant pressure in a manner similar to the adaptation of excitable tissues to the flow of a constant current   Repetitive applications of pressure may be regarded as repeatedly stretching the nerve and stimulating it, perhaps by physical alteration of its polarized membranes   As in the case of repetitive, non-injurious stimulation of excised nerve we might expect impulses to be set up in response to each stimulation, provided the intervals between stimuli do not exceed the refractory period of the nerve   Such repetitive activity of excised nerve has been found to be maintained over long periods of time

The failure of response of the cutaneous endings to the repeated stimulations of the air jet has been referrred to above as sensory adaptation   The receptive nerve fiber is surrounded by epithelial

cells which are distorted by the mechanical stimulus and it is not unreasonable to suppose that substances may be released from the cells surrounding the fiber as a result of their distortion   Such substances accumulating about the fiber may progressively lower its excitability, ultimately silencing it by increasing its threshold to stimulation

Adrian, Cattell, and Hoagland (1931) reported that if the surface layers of frog's skin were scraped away the tactile responses (nerve impulses) ceased for a time but eventually returned   Feng (1933) confirmed this and extended the observations, showing that the failure was due to the release of potassium from broken epithelial cells, which temporarily reduced the excitabilities of the endings   Feng found that the temporary abolition of responses occurs not only in the region scraped, but also in adjacent unscraped regions   Irrigation with Ringer's solution caused the responsiveness to return   Samples of scraped and unscraped frog's skin were applied to the underside of the skin nerve preparation   Inhibition of response occurred only when the preparation was in contact with scraped skin   The inhibitory effect was found not to be peculiar to damaged skin alone   Crushed frog tissues in general were found to inhibit the tactile nerve impulses   The effect was shown not to be due to pH or to osmotic pressure changes

Ringer's solution with about ten times the normal potassium content was also found to inhibit responses, as did solutions made up of ashed tissue extracts   Potassium assays were made of the inhibiting tissue extracts which were found to inhibit to about the same degree as solutions containing equal amounts of potassium

This work is suggestive in connection with the quantitative analysis of the spread of effects of operational injury along the lateral line receptors of fishes (Hoagland, 1932-33b)   A depressing action on spontaneously discharged nerve impulses from the neuromasts was found for a distance of ± 2 cm from the region of operation, the inhibitory effect decreasing with the distance from the incision   The high mobility of potassium ions is consistent with the notion of its production of such injury effects

Blinks (1933) has reported the inhibition of recovery in the presence of potassium after the passage of action currents in cells of *Nitella* and

Chara   He suggests that the effect is due to a lowering of acidity within the cells by the entrance of potassium in the form of a base (For a review of the relation of potassium to bioelectric currents in plant cells *cf* also Osterhout, 1931)

The following facts appear to be consistent with the hypothesis that adaptation of tactile receptors may be due to the liberation of potassium from surrounding epithelial cells when pressed upon, reducing the excitabilities of the nerve endings to direct mechanical stimulation

III

(*a*) Experiments involving brief durations of the air puff stimulus show that the receptor can follow frequencies of stimulation of the order of 350 per second for a short time ($\pm$ 0 5 second) at room temperature   The brief intervals between stimuli at these high frequencies may force the nerve to conduct in its relative refractory period, giving axon potentials which may be reduced 50 per cent below normal (Cattell and Hoagland, 1931)   The brief time relations involved imply that the stimulus effects the fiber directly, without the mediation of chemical mechanisms

(*b*) Adaptation to a jet of air at constant pressure stimulating at 140 impulses per second was measured with eleven single-fiber preparations   The time of complete adaptation was recorded with a stop-watch by observing the failure of impulses on a viewing screen After complete failure of impulses a jet of Ringer's solution was passed from a pipet across the *under* side of the skin through an incision a few millimeters from the ending   The solution drained away through a second skin incision below the ending   The washing in all but two cases effected a rapid recovery of the ending—impulses being produced from the previously adapted receptor, not only during the washing, but for about half the normal adaptation time after cessation of the current of Ringer's solution

It was thought that the apparent recovery might have been due to slight elevation of the skin towards the stimulating nozzle by the entering solution, thus rendering the stimulus more effective   Accordingly the skin was lifted slightly by the tip of the pipet and also by blowing air through the pipet against the under side of the skin   In

a few cases slight augmentation of the response seemed to result but to no such degree as was observed when the skin was washed with the current of Ringer's In six experiments isotonic KCl was used to

TABLE I

Stimulation frequency = 140/second

Time required for complete failure of impulses with different durations of recovery between determinations

60 sec recovery period	30 sec recovery period	15 sec recovery period	Approximately 3 sec. recovery period
sec	sec	sec	sec
15 2	15 0	8 2	2 2
15 0	14 6	7 2	1 8
14 8	15 4	7 6	3 0
15 6	14 8	8 4	1 2
15 4	15 2	8 8	2 0
Means 15 2	15 0	8 0	2 0

Immediately after complete adaptation with the intermittent stimulus continuing the skin was washed with Ringer s solution for a period lasting 8 ± 2 sec.

Time from start of washing to second complete adaptation
sec
20 2
25 8
18 0
26 0
23 8
Mean 22 8

After washing with isotonic KCl (30 sec. recovery period) adaptation time
sec
6 0
2 0
0 0

Repeated washing with Ringer s solution produces complete recovery

wash the under side of the skin This qualitatively caused a hasten ing of adaptation Washing with Ringer's solution after the failure due to KCl hastened the recovery Table I shows the results of one experiment

(c) The speed of adaptation is increased with increase in the ratio of $\dfrac{\text{Stimulating time}}{\text{Time of rest between stimuli}} = S/R$ as determined with a series of notched discs used to interrupt the air jet (Hoagland, 1932-33 a)   In some preparations the velocity constant of the process of adaptation was found to be directly proportional to S/R, in others it was proportional to its logarithm

This is consistent with the notion that potassium released from surrounding cells reduces the excitability of the nerve fiber—the greater the value of S/R the more rapidly potassium would be released and the faster would be the sensory adaptation   The excitability of nerve depends on the ratio of potassium inside the fiber (K_i) to potassium outside (K_o), where $\dfrac{K_i}{K_o}$ is normally about 10 (cf Hill, 1932).   Potassium released from surrounding epithelial cells would tend to decrease this ratio by raising K_o, thus reducing the excitability of the nerve fiber   For a review of the relation of the excitability of muscle to its potassium content cf Needham (1932, chapter 7)

In nine out of fifteen preparations studied the frequency of response of the ending to the air puff stimulus was found to decline logarithmically with time   This is consistent with the notion that diffusion of potassium may be the determining factor in producing adaptation   In six out of fifteen cases the frequency declined hyperbolically and at a more rapid rate than in the other experiments   This digression from the logarithmic decline may have resulted from the fact that in some preparations the recovery mechanism maintaining $\dfrac{K_i}{K_o}$ may have been retarded, thus facilitating the accumulating potassium and producing adaptation

(d) A series of preliminary experiments on adaptation as a function of temperature has been carried out by the writer in the laboratory of W J Crozier at Harvard   A satisfactory quantitative analysis of these results is not yet complete   Qualitatively, however, adaptation is found to be faster the lower the temperature   This may be due in part to the fact that at lower temperatures the skin appears to be less flexible, as judged by its movement in response to the stimulus, thus rendering the pressure less effective and reducing the stimulus to

nearer the threshold value for the receptor Any increase of potassium ions from epithelial cells in the vicinity of the fiber would tend to be more effective in reducing the excitability of the nerve owing to reduction of the effective stimulus, despite the fact that presumably less potassium would be released at lower temperatures It is also probable that at low temperatures chemical recovery processes of the nerve maintaining the ratio $\frac{K_i}{K_o}$ would be reduced, resulting in an increased rate of failure during repeated excitations

Table II shows that the frequency of the functional occurrence of antidromic tactile endings increases with temperature This may be the result of greater pliability of the skin at higher temperatures resulting in more vigorous movements of the stimulated skin and hence

TABLE II

Mean experimental temperature ≈ 0 3 approximately	No. of frogs	Total No antidromic endings	Antidromic endings per frog
c			
7 2	15	24	1 6
11 1	10	22	2 2
15 0	11	28	2 5
18 6	4	12	3 0
24 7	6	24	4 0

more effective stimulation Thus receptors of high thresholds of activation may be stimulated more effectively at higher temperatures The increase in the number of observed responsive antidromic endings with temperature may also be accounted for in terms of facilitation of chemical factors involved in regulating thresholds of excitability of the nerve endings, i c , in producing augmentation of the ratio $\frac{K_i}{K_o}$

Throughout the course of the experiments it has been repeatedly observed that the degree of movement of the skin makes considerable difference in the time of adaptation for the same preparation A difference of 100 per cent in the time of complete adaptation may be obtained by merely altering the angle at which the stimulus impinges upon the skin In quantitative studies of the single endings it has

therefore been found essential to keep the position of the nozzle with respect to the skin rigidly fixed throughout work with a particular preparation

(e) Cattell and Hoagland (1931) found that if an uninterrupted jet of air was applied gradually for approximately 30 seconds to a single
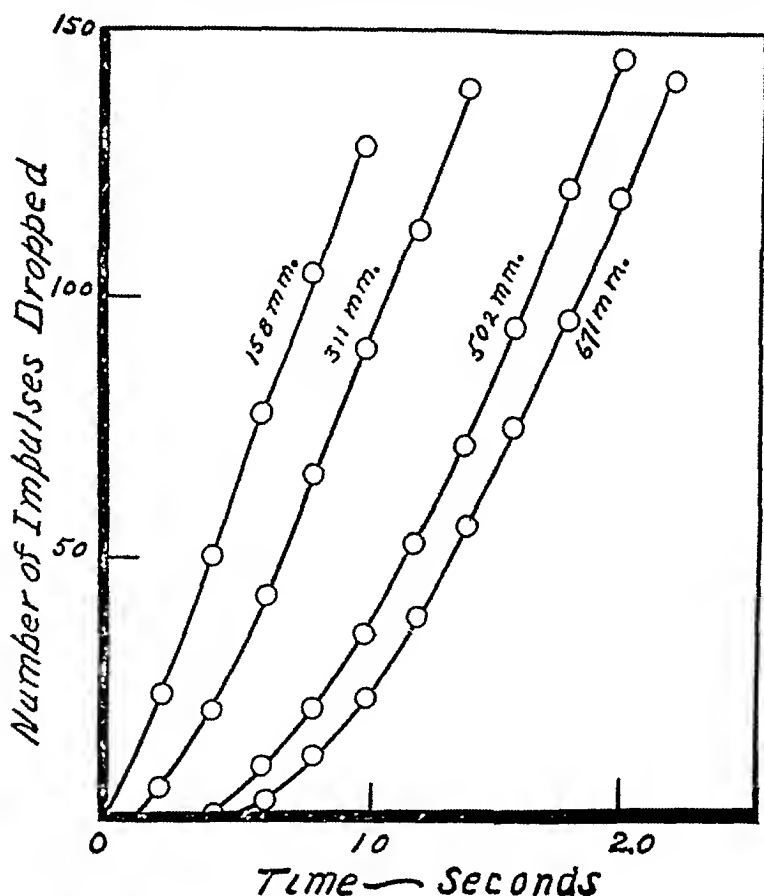
Fig 1 Adaptation curves for a single ending at different pressures (mm Hg) The data were obtained by counting, from photographs, the number of impulses dropped after the beginning of stimulation at zero time The stimulation frequency was 140 per second

receptor, setting up no nerve impulses, the receptor nevertheless became by this treatment, adapted to a normally effective intermittent air jet applied immediately afterward

This finding is consistent with the proposed general hypothesis—

the slowly increasing pressure is unable to excite the fiber but it may nevertheless cause leakage of potassium from the epithelial cells Thus the ratio of $\dfrac{K_i}{K_o}$ of the nerve fiber may be reduced by the increase in K_o so that a subsequent interrupted stimulus becomes ineffective

(f) It was also reported by Cattell and Hoagland (1931) that stimu lation of a part of a given skin area supplied by a single branching axon produced adaptation, not only in the stimulated area, but also to a second intermittent stimulus applied immediately after the first, to an adjacent area several millimeters distant, supplied by the same branching axon In terms of the present hypothesis this is to be expected owing to the diffusion of potassium ions, released from epithe lial cells by the stimulus, to the neighboring area (cf discussion of Feng's experiments)

(g) Recent experiments indicate, in general, that adaptation of the tactile receptors is faster at lower pressures Fig 1 shows this relation Lowering the pressure decreases the movement of the skin and hence the adequacy of the stimulus, so that the stimulus approaches more nearly its threshold value Presumably less potassium will also be released from the epithelial cells, producing less effect on the excita bility ratio $\dfrac{K_i}{K_o}$ However, if the adequacy of the stimulus declines rapidly with pressure, even a small increment in K_o may be sufficient to produce rapid sensory adaptation

SUMMARY

Studies of axon potentials set up by pressure stimuli applied to single cutaneous receptors in frog's skin indicate that the mechanical stimulus excites the free nerve endings directly

Adaptation to constant pressures or to intermittently applied pres sures (failure of the response) may be due to the reduction of excita bility of the nerve endings by potassium released under the pressure from surrounding epithelial cells

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THE ABSORPTION OF ULTRA VIOLET RADIATION BY CRYSTALLINE PEPSIN

WORK BY FREDERICK L. GATES*

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In a previous paper (Gates, 1933–34) the absorption spectrum of Northrop's crystalline pepsin was shown to have a characteristic shape similar to that of certain amino acids (Gates, 1928), corresponding to the essential protein nature of the enzyme (*cf* Northrop, 1929–30, 1932–33*a*, Sumner, 1933)

As a possible step toward more definite knowledge of enzyme structure it is of interest to know whether the destruction of pepsin activity varies in the same way with different wave lengths as does the absorption of energy in the different parts of the spectrum

The changes produced in pure crystalline pepsin by ultra violet radiation were studied in two principal ways (1) by direct determination of the absorption spectrum of pepsin inactivated by ultra-violet energy, and (2) by measuring the activity of pepsin solutions after irradiation with different bands of wave lengths of the mercury vapor arc spectrum As in the previous work (Gates, 1933–34), all of the pepsin preparations used were obtained through the kindness of Dr John H Northrop

I

The Absorption Spectrum of Crystalline Pepsin Inactivated by Ultra Violet Radiation

Early absorption tests made upon different preparations of pepsin after irradiation with the total radiation from a quartz mercury vapor arc and with single wave lengths obtained by means of a crystal quartz monochromator (Gates, 1929–30), indicated that definite

changes occurred in the absorption curve of pepsin solutions exposed to high incident energies   These changes were especially marked in the region of the spectrum between 2400 and 2750 Å u

Numerous tests carried out on different preparations of pepsin (of varying stages of purification) produced essentially similar curves of absorption at corresponding incident energies   Only tests made on a highly purified preparation of crystalline pepsin (Lot 4) are considered here

EXPERIMENTAL

Highly purified crystalline pepsin (Lot 4) having the characteristics $2\times$ crystalline pepsin in м/10 sodium acetate, pH 5 0, protein nitrogen—21 4 mg per ml , (P U)$_{ml.}^{Hb}$—4 4, was diluted to 1 in 25 with м/10 sodium acetate, pH 5 0 (by quinhydrone electrode)   Two 25 ml samples of this solution were pipetted into 9 cm Petri plates covered with cellophane, the control was also covered with a plate of soda glass (1 55 mm thick)   Exposure was at 30 cm from an air-cooled horizontal quartz mercury vapor arc operated at 67 volts and 5 5 amperes During the exposures the temperature of the specimens was maintained between 20 and 22°C by a bath of ice water

At intervals of 20, 60, 180, and 360 minutes 5 ml samples were removed for tests of the pepsin activity by the hemoglobin method of Anson and Mirsky (1932–33), and for absorption tests   The latter tests were made as previously described by Gates (1930–31, 1933–34), using a quartz sector photometer (Judd-Lewis, 1919, 1921) and a large quartz spectrograph   The pepsin solution, as irradiated, along with a control solution of the solvent (м/10 sodium acetate buffer, pH = 5 0) was placed in micro-Baly photometer tubes in the path of twin beams of light from the tungsten-iron spark source   Variation of the length of the solution in conjunction with the sector vanes of the photometer permitted a wide range in the intensity of the transmitted energies

From the spectrograms thus obtained the absorption curves were plotted in terms of the molecular extinction coefficients as a function of wave-length (Fig 1)

The curves of Fig 1 show a progressive change in the S shape of the absorption curve with increasing periods of exposure   For analysis the curves may be divided into four regions   (1) the slope upward between 3100 and 2850 Å u , (2) the peak at 2775 Å u , (3) the valley at 2500 Å u   (4) the upward slope from 2475 to 2300 Å u   Irradiation did not greatly affect the peak at 2775 Å u , or the final slope between 2475 and 2300 Å u   but the absorption in (1) broadened into the near ultra-violet and (3) filled up

Points plotted from data secured on unexposed control solutions and on specimen solutions exposed for 6 hours under soda glass are in close agreement with those of solution A_1 exposed for 20 minutes. This indicates that relatively long exposures to radiation of shorter wave-
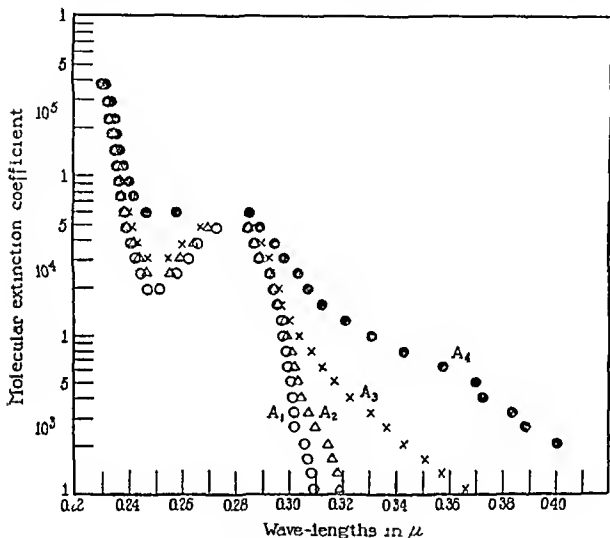
Fig 1 Curves showing the absorption spectra of solutions of pure crystalline pepsin (5 65 mg per ml) in N/10 sodium acetate buffer, pH 5 0 irradiated for different periods of time at 30 cm from an air-cooled horizontal quartz mercury vapor arc operated at 67 volts and 5 5 amperes Clear circles represent points obtained on solutions irradiated 20 minutes triangles 60 minutes, crosses 180 minutes and solid circles, 360 minutes

lengths than those transmitted by soda glass are required to change appreciably the absorption spectrum of the pure enzyme

Absorption tests on solutions of pepsin (at the same dilution and pH as the above) with higher energies gave results similar to those of

Fig 1 with a further rise in the curve at 2500 Å u and with a further broadening out of the absorption into the near ultra-violet and blue region of the spectrum

Crystalline Pepsin Inactivated by Heat

To test for any change in the absorption spectrum of pepsin due to heat, a pure preparation of crystalline pepsin (21 4 mg protein nitro-
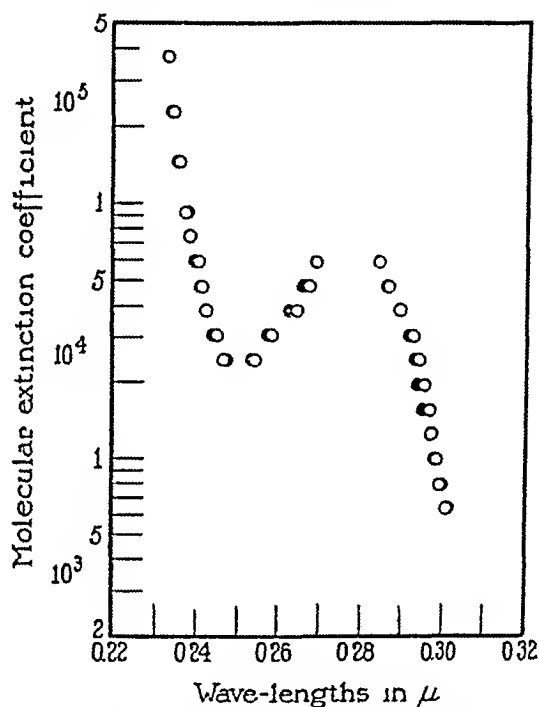
FIG 2 The absorption spectrum of pepsin inactivated by heating to 65°C for 5 minutes, compared with the normal absorption spectrum   Clear circles represent points on the ultra-violet absorption curve of pure crystalline pepsin (0 1412 mg per ml , pH 4 9) at 100 per cent activity   Solid circles represent points on the absorption curve of the same pepsin solution inactivated by heat, activity, 43 5 per cent

gen per ml in N/10 sodium acetate buffer, pH 5 0) was diluted 1 in 25 with N/100 HCl [1]   A 5 ml sample of this solution was heated for 5 minutes at 65°C while an equivalent sample was held as a control

[1] Later tests of the pH of the diluted pepsin solution indicated that the pH at the time of the absorption tests was probably between 4 9 and 5 0   The N/100 HCl had probably deteriorated

Both samples were then diluted 1 in 40 with N/100 HCl and their activity was tested by the hemoglobin method and read against the tyrosine standard set at 20 On the basis of the undiluted pepsin the heated sample showed an activity of $(P U) = 1 18$, or a 56 5 per cent loss in activity in the heated sample

Both samples were studied spectrographically in the ultra violet with the Judd Lewis photometer and micro Baly tubes as described previously On plotting the absorption curves (Fig 2) it is evident that there is not enough difference between the two to be significant

II

The Inactivation of Crystalline Pepsin by Ultra Violet Radiation[2]

It is essential that a correlation be made between the changes in the absorption spectrum of crystalline pepsin as a result of irradiation and changes in the activity of the enzyme To obtain such data tests of the proteolytic activity of pepsin after irradiation were made

Early tests of the activity of pepsin solutions by viscosity measurements (Northrop, 1932–33a) on the rate of digestion of edestin after irradiation of the enzyme solutions, showed that exposure to narrow bands of wave lengths for 1 and 2 hours was not sufficient to produce any appreciable inactivation, but that 35 minute exposure at 30 cm to the total radiation from an air cooled horizontal quartz mercury vapor arc was sufficient to inactivate the pepsin at dilutions of 0 002 mg protein nitrogen per ml

Tests of the activity of the highly purified pepsin solutions used in obtaining the absorption curves given in Fig 1 were made by the hemoglobin method of Anson and Mirsky (1932–33) For the activity determinations the pepsin solutions (as irradiated, see above) were further diluted to 1 in 40 with N/100 HCl to give a pH of 2 7

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[2] The results of a number of tests on the activity and the absorption spectra of a number of preparations of crystalline pepsin of varying purities at different concentrations and at different levels of pH, agree essentially with those reported here and are therefore omitted to avoid undue repetition In these tests the activity of the irradiated pepsin solutions was tested by several methods the dissolving action on gelatin (Northrop and Hussey, 1922–23 Northrop 1929–30) and on gelatin films (Gates, 1927, 1930), and the rate of digestion of casein and of 5 per cent edestin as measured by the viscosimetric method (Northrop 1932–33 b)

1 ml of this 1/1000 dilution was used in each test (Table I), blank tests on hemoglobin using 1 ml of м/100 HCl in place of pepsin yielded no coloration with the phenol reagent (Folin and Ciocalteau, 1927)

All irradiated and control specimens were clear in color, excepting the 3 and 6 hour exposure specimens (A_3 and A_4) which showed a yellow coloration A_4 had the odor of fresh urine or stale straw The results of these tests, with the calculated pepsin activity, $(P U)_{ml}^{Hb}$, are given in Table I

TABLE I

Pepsin Activity Measured by Titration with Hemoglobin (Anson and Mirsky, 1932–33)

Samples irradiated with the total radiation from a horizontal quartz mercury vapor arc at 30 cm , operated at 67 volts and 5 5 amperes

Specimen	Period of irradiation	Colorimeter readings (standard set at 20)				$(P U)_{ml}^{Hb}$ $\times 100$	Activity
					Average		
Control	*min*						*per cent*
	0	11 8	11 8	11 8	11 8	1 5	
A_1	20	11 2	11 2	11 2	11 2	1 6	100*
B_1	20	11 2	11 4	11 2	11 2	1 58	
A_2	60	14 0	14 0	14 1	14 0	1 24	79 5
B_2	60	11 4	11 5	11 4	11 4	1 56	
A_3	180	16 2	16 3	16 4	16 3	1 04	68 5
B_3	180	11 8	11 6	11 6	11 7	1 52	
A_4	360	31 4	31 3	31 3	31 3	0 478	32 4
B_4	360	11 9	11 9	11 9	11 9	1 49	

* Since A_1 and its control exposed under glass, B_1, are close together and B_2, B_3, and B_4 fall off gradually from the first figures and are all above or equal to the unexposed control, it is advisable to regard 1 59 as representing 100 per cent and to calculate the per cent loss in activity on this basis

On plotting the percentage pepsin activity semilogarithmically against the period of irradiation a straight line is obtained, indicating that the pepsin is inactivated in a first order reaction with a one-quantum relationship, assuming the energy flux to be constant Northrop (1933–34) found a similar relationship between the energy

and the rate of inactivation for pepsin solutions at different pH values (see also Collier and Wasteneys, 1932, Hussey and Thompson, 1923–24)

Since the activity tests show a mass reaction (one quantum) curve, presumably a single reaction takes place, and the successive curves $(A_1 - A_4)$ represent mixtures of the pre and post irradiated substance On this assumption the reciprocals of the valley points at 2500 Å u should bear some relation to the change in activity of the pepsin The successive values are given in Table II with the respective pepsin activities for comparison

TABLE II

Pepsin Activity Estimated from the Reciprocals of the Ordinates of the Absorption Curves of Fig 1 at Wave Length 2500 Å u for Pepsin Solutions Irradiated for Varying Periods of Time

Absorption curve	Curve ordinate at 2500 Å u	Reciprocals of ordin tes	Unch ged pepsin	Pepsin activity
			per cent	*per cent*
A_1	1 9	0 526	100	100
A_2	2 34	0 425	80 8	79 5
A_3	2 85	0 351	66 7	68 5
A_4	5 68	0 176	33 4	32 4

* On the assumption that the reciprocal of the ordinate in Curve A_1 represents unchanged pepsin at 100 per cent activity, the other activities are in the ratios given in Column 4 of Table II

Since the actual ratios of pepsin activity found by the hemoglobin method are given in Column 5 of Table II, the agreement with the values obtained from the reciprocals of the absorption ordinates (Column 3) suggests that the destruction spectrum of pure crystalline pepsin coincides with its absorption spectrum, at least in this region of the ultra violet If we assume the final substance to have a straight line absorption between 2850 and 2350 Å u the curve would cross the 2500 Å u line at about 20 0 This point, considered as above, corresponds to an activity of 10 per cent

Although the agreement between the values of Columns 4 and 5 of Table II is close, certain relations between the pepsin concentration and the absorption coefficient should be kept in mind

If we let C = the concentration of pepsin at any time t
and C_o = the original concentration of pepsin
then $C_o - C$ = the reaction products at time t
Now letting β = the extinction coefficient of pepsin,
γ = the extinction coefficient of the reaction products,
μ_x = the absorption coefficient of the mixture at time t,
and μ_o = the initial absorption coefficient,

then

$$\mu_x = C\beta + (C_o - C)\gamma \tag{1}$$

from which we get

$$C = \frac{C_o\gamma - \mu_x}{\gamma - \beta} \text{ (since } \gamma > \beta) \tag{2}$$

and

$$C = C_o\left(\frac{\gamma}{\gamma - \beta}\right) - \left(\frac{1}{\gamma - \beta}\right)\mu_x \tag{3}$$

or

$$C_o - C = k(\mu_x - \mu_o) \tag{4}$$

Equation (4) implies a linear relationship between the amount of inactivation and the change in the absorption coefficient, and hence is not in accordance with the data of Column 5, Table II Two explanations may be advanced to explain this divergence (1) error in the determination of the ordinates from the curves of Fig 1 at 2500 Å u , and (2), a further change in the reaction products resulting in an increased absorption with increasing periods of exposure

III

The Destruction Spectrum of Crystalline Pepsin

To determine the relation existing between radiant energy absorbed by the pepsin and the amount of inactivation, a series of tests was made in which pure crystalline pepsin from the same preparation already described was diluted to 1 in 500 with N/100 HCl, at pH 2 06, and exposed in two cells ($5 \times 5 \times 23$ mm) cut in a glass block and faced by a 2 mm quartz plate, by means of a large quartz monochromator (Gates, 1929–30) Specimens were irradiated for different periods at four different calibrated scale settings of the monochromator At

each of these settings the exit slit of the monochromator was so ad justed that a band of known wave lengths was incident on the exposure cell   To obtain the energy absorbed by the solution the intensity of the incident and transmitted radiation was read with a sensitive four-junction thermopile and a sensitive Leeds and Northrup galvanometer   During the exposure the control cell was cut off from direct radiation by the exit slit of the monochromator   Due to the very small free surface there was very little evaporation from the test
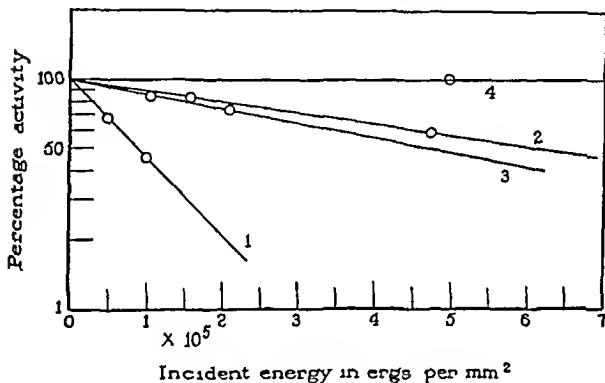
FIG 3 The inactivation of pure crystalline pepsin, pH 2 06 by ultra violet radiation at different parts of the spectrum, Curve 1 the band of wave lengths between 2300 and 2400 Å u Curve 2 2425 to 2570 Å u , Curve 3, 2640 to 2820 Å u , Curve 4, 2860 to 3131 Å u

samples   If evaporation could be detected the specimen was made up to the correct volume (0 575 ml) by addition of N/100 HCl

After irradiation specimens were removed from the exposure cell with capillary pipettes, placed in conical centrifuge tubes, measured with 1 ml pipettes, and an equal quantity of N/100 HCl was added to give a final dilution of 1/1000 of the original pepsin solution

The exposed, control, and stock specimens were tested for activity by hemoglobin proteolysis, using one half quantities throughout

After mixing, the specimen was kept at 35 5°C for 5 minutes and then read against a carefully prepared tyrosine standard obtained from Dr P A Levene   During the exposures the temperature of the specimens varied between 25° and 27°C

TABLE III

Protocol of Tests of the Relation between Absorbed Energy and the Amount of Inactivation of Pepsin (pH 2 06) at Different Bands in the Ultra-Violet

Point wave length	Specimen sample	Energy		Exposure time	(P U) Hb for original No 4 /ml	Pepsin activity	Energy required to inactivate 50 per cent of pepsin		α
		Incident	Absorbed				Incident	Ab sorbed	
$\lambda\,u$		*ergs/mm*	*ergs/mm²*	*min*		*per cent*	*ergs/ mm²*	*ergs/ mm²*	
2357	A_1	$4\ 96 \times 10^4$	$4\ 26 \times 10^4$	45	2 14	68 6			
(2300–	B_1				3 12				
2400)	A_2	$9\ 92 \times 10^4$	$8\ 52 \times 10^4$	90	1 43	45 6	87,000	77,500	14 $\times 10^4$
	B_2				3 12				
2509	C_1	$15\ 7 \times 10^4$	$7\ 5 \times 10^4$	60	2 58	82 7			
(2425–	D_1				3 12				
2570)	C_2	$47\ 3 \times 10^4$	$22\ 5 \times 10^4$	180	1 84	59 0	617,000	305,000	1 97 $\times 10^4$
	D_2				3 12				
2719	Γ_1	$10\ 4 \times 10^4$	$5\ 3 \times 10^4$	60	2 18	84 4			
(2640–	G_1				2 58				
2820)	C_0*				2 58		468,000	230,000	
	Γ_2	$20\ 8 \times 10^4$	$10\ 6 \times 10^4$	120	2 00	74 0			2 62 $\times 10^4$
	G_2				2 70				
2930	H_1	$49\ 9 \times 10^4$	$11\ 8 \times 10^5$	120	2 60	98 5			
(2860–	I_1				2 64				
3131)	H	$49\ 9 \times 10^4$	$11\ 8 \times 10^5$	120	3 12	100 0			
	I_2				3 12				

* Flask control

From calculations of the relative energies of the wave-lengths included in each of the four bands of the spectrum used, the relative absorption of the pepsin solutions at these wave-lengths, the relative transmission of energy, and the relative efficiency of each wave-

length in contributing absorbed energy, it was found that the light
acted practically as though concentrated at the following points

Monochromator setting	11 0	9 5	7 8	6 5
Point wave length in $4 u$	2357	2509	2719	2930
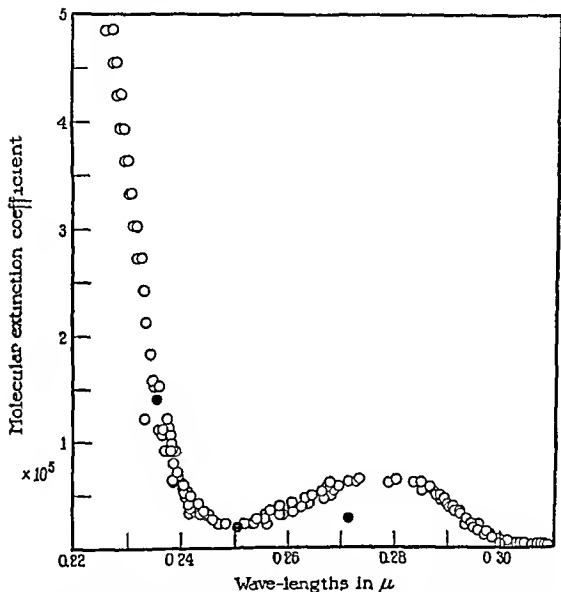
FIG 4 Comparison of the destruction spectrum of Northrop s crystalline pepsin
with its absorption spectrum   Clear circles represent points obtained with a pure
crystalline pepsin preparation (0 2376 mg per ml) in N/100 HCl, pH 2 54 on the
normal absorption curve   Solid circles represent the reciprocals of the energy /100
required to inactivate 50 per cent of the pepsin (0 1412 mg pepsin per ml.) in
N/100 HCl, pH 2 06, plotted against the point wave length   (See text)

Tests of the pepsin activity translated into percentage of activity
remaining give straight lines when plotted semilogarithmically against
the incident energy (Fig 3), indicating the same one quantum rela

tionship already suggested   Protocols of these experiments are summarized in Table III

If we assume that the inactivation efficiency of the various wave-lengths is proportional to their respective absorption coefficients, the ordinates of the destruction curve may be calculated from the relation

$$\frac{\alpha_2}{\alpha_1} = \frac{(\ln\ C_o/C)_2}{(\ln\ C_o/C)_1} \frac{(\imath\ t)_1}{(\imath\ t)_2}$$

where α_1 represents the extinction coefficient determined by the destruction at a reference wave-length λ_1 and α_2 represents the coefficient at any other wave-length λ_2 C_o and C represent the concentration of active pepsin at the beginning and after a period of exposure t, and $\imath$ is the intensity of the incident radiation (cf Kubowitz and Haas, 1933)

Calculation of the number of quanta absorbed (2×10^{14}) and the number of molecules of pepsin inactivated ($5\ 6 \times 10^{14}$) at the point wave-length 2357 Å u , indicates that the one-quantum relation holds at this wave-length under the conditions of the experiment   Since most of the incident energy is absorbed at 2357 Å u , the extinction coefficient has been substituted for α_1 as the point of reference in calculating the other values of α by the above equation (Column 10, Table III)

These values when plotted as a function of wave-length (points represented by solid circles in Fig 4) give the approximate curve of the destruction spectrum of pepsin

<div align="center">IV</div>

<div align="center">DISCUSSION</div>

Comparison of the destruction spectrum of pure crystalline pepsin with the absorption spectrum (Gates, 1933–34) reveals an essential agreement in the location of the maxima and minima of the two curves for the several bands of wave-lengths tested   However, it should be noted that an exact agreement does not exist between the two curves at 2719 Å u , with the present method of comparison   Furthermore, a comparison of the incident and absorbed energies required to inactivate 50 per cent of the pepsin shows that considerably more energy must be absorbed at the wave-lengths 2509 Å u and 2719 Å u to

inactivate a given amount of pepsin than would be required if a quantum yield of one (as indicated at 2357 Å u) holds for each of these wave lengths   From the logarithmic rate of inactivation (Fig 3), and from the change in the absorption spectrum as the reaction proceeds with increased exposures to the radiation (Fig 1), it is prob able that the differences observed may be due to varying absorption of energy by the products of the reaction

Kuhowitz and Haas (1933) describe a similar correspondence between the destruction spectrum and the absorption spectrum of urease (Sumner, 1926) (with the exception of a discrepancy in the values of the absorption coefficients at the wave length 254 mμ)

As previously noted for the absorption spectra (Gates, 1933–34), there is considerable agreement between the destruction spectrum of pepsin at this level of pH (2 06) which Northrop (1933–34) has shown to be in the range of optimum inactivation by ultra violet radiation, and the destruction spectrum of urease

In connection with the protein nature of pepsin it is of interest to note the parallelism of the observed increase in the total absorption of energy by pepsin inactivated by ultra violet radiation with the increase in total absorption and loss of the specific absorption band near 265 mμ by tuberculin after ultra violet irradiation (Spiegel Adolf and Seibert, 1933)

SUMMARY

Determination of the absorption spectra of pure preparations of Northrop's crystalline pepsin inactivated by irradiation with ultra violet light shows that the total absorption in the ultra violet region of the spectrum increases with the degree of inactivation   This increase is especially marked between 2400 and 2750 Å u   The rate of photoinactivation is shown to be sensitive to changes in pH, increasing with lower values, and evidently bears a one quantum relationship to the energy flux   Tests of the rate of inactivation of pepsin exposed to several different bands of the ultra violet spectrum, in relation to the absorbed energy, indicate that the destruction spectrum of the enzyme agrees essentially with its absorption spectrum and is similar to that of urease

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THE TEMPERATURE COEFFICIENT OF INACTIVATION OF CRYSTALLINE PEPSIN BY ULTRA VIOLET RADIATION

WORK BY FREDERICK L GATES*

(*From the Laboratory of General Physiology, Harvard University Cambridge*)

In determining the destruction spectrum of Northrop's crystalline pepsin (Northrop, 1929–30, 1932–33, 1933–34, Gates, 1933–34) and the changes in the absorption spectrum with inactivation of the enzyme by ultra violet irradiation, it was noted that increase in the temperature to 65°C for 5 minutes resulted in the inactivation of more than 50 per cent of the pepsin The question remains as to how fluctuations in temperature will affect the rate of inactivation produced by the absorbed radiant energy

From the nature of the reaction, i e the indicated single quantum relationship between the inactivation of the pepsin and the incident energy (Gates, 1934–35), it might be assumed that the process was direct or physical rather than chemical, and that a low temperature coefficient approaching unity would be obtained To test this point and also to determine the effect of temperature fluctuations during the exposure period, experiments were performed in which crystalline pepsin (Lot 4) in sodium acetate buffer, pH 5 0, was diluted to 1 in 500 with M/100 HCl, pH 2 1, and exposed for different periods at several temperatures to radiation of wave length 2357 Å u

The solutions and controls were exposed in the quartz faced glass cells previously described (Gates, 1933–34), in a large quartz mono chromator (Gates, 1929–30), with the temperature maintained at the desired level by a glass water chamber constructed on the side of the cell away from the exit slit of the quartz monochromator Into this

chamber a constant stream of water flowed from a storage vessel which could be heated by an electric heater or cooled by ice cubes During the exposure the control cell was protected from direct radiation by the exit slit of the monochromator
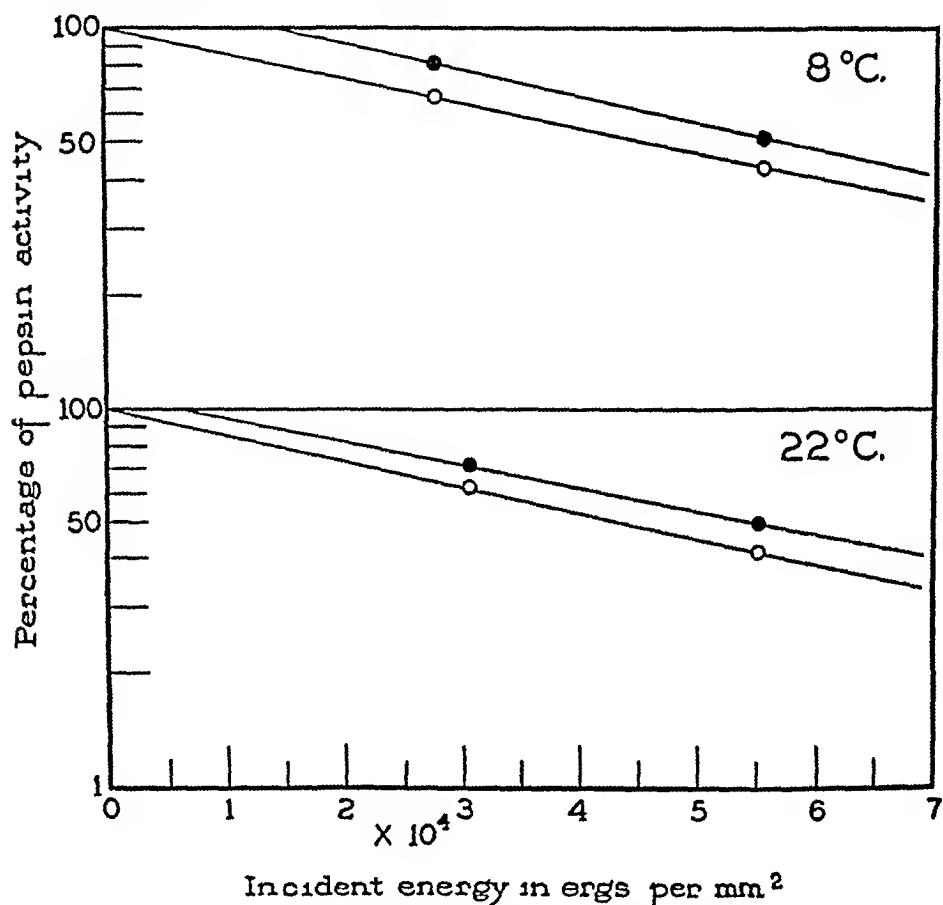
Fig 1 The rate of inactivation of crystalline pepsin, at pH 2 1, by ultra-violet irradiation at 2537Å u , for different periods of time, at two different temperatures Solid circles represent calculations on the basis of 100 per cent activity for the corresponding controls   Clear circles represent points calculated on the basis of 100 per cent for the flask control

After irradiation the exposed specimens and their controls, along with flask controls, were tested for pepsin activity by the hemoglobin method of Anson and Mirsky (1932–33)

The protocol of tests carried out at 22° and 8°C is given in Table

I   The pepsin activity for each specimen and control has been cal
culated by the equation used by Anson and Mirsky (1932–33, p 61)
To determine the inactivation due to the absorbed ultra violet energy
alone, the per cent activity has been calculated on the basis of 100
per cent activity for each control

TABLE I

*Experiments on the Inactivation of Pepsin Solutions by Ultra Violet Irradiation
at 2537 Å u , pH 2 1, at Two Temperatures   Pepsin Activity Determined
by the Hemoglobin Method*

Speci men	T	Period of exposure	Energy			Colorim eter reading average	$(P U_{mL}^{Hb} \times 100)$	Pepsin activity		Energy to Inactivate 50 per cent of pepsin Incident
			Inci dent	Trans mitted	Absorbed					
			ergs/ mm²	ergs/ mm²	ergs/mm²			per cent	per cent	
A₁	C	37 24″	27 800	7 850	19 950	14 9	1 152	80 2	66 7*	
B₁	8	—				12 3	1 438	100		56 600 45 300*
A₂		74 48″	55 600	15 700	39 900	22 0	0 739	51 0	42 8*	
B₂		—				12 2	1 449	100		
C₀		Flash control				10 4	1 728		100*	
D₁		37 24	30 880	8 960	21 920	15 9	1 080	71 8	62 5*	
E₁	22	—				10 7	1 675	100		54 700 44 000
D₂		74 48	55 200	16 080	39 12	22 0	0 739	49 2	42 8*	
E₂		—				11 8	1 505	100		

* On the basis of flash control = 100 activity

On plotting the per cent activity of each specimen at the given
temperature as a function of the incident energy, a straight line may
be drawn through the points (Fig 1) according to the one quantum
relation found in previous tests (Gates, 1933–34, Northrop, 1933–34)
For the values calculated on the basis that each corresponding control

represents 100 per cent pepsin activity (solid circles) the line does not strike the origin, due probably to stray light and other external factors   The temperature coefficient on this basis, obtained by taking the reciprocal of the energy ratios for a 10° change in temperature (Column 11, Table I), was found to be 1 024   If we use the activity of the flask control as 100 per cent activity the resultant curves strike the origin (clear circles) and the temperature coefficient is 1 020 In either case the temperature coefficient is so near to unity that a direct inactivation of the enzyme by the absorbed energy is indicated Hussey and Thompson (1925–26) found a similar situation for the inactivation of pepsin by radiations from the radioactive products in equilibrium with radium emanation

CONCLUSION

Determinations of the temperature coefficient of inactivation of pure crystalline pepsin solutions by ultra-violet irradiation give values very close to unity (1 02)

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THE ACCUMULATION OF ELECTROLYTES

VII Organic Electrolytes

Part 2*

By A G JACQUES

(From the Laboratories of The Rockefeller Institute for Medical Research)

The first part of this paper demonstrates a marked preponderance of inorganic cation equivalents in saps from the leaves of *Rheum*, *Rumex*, and *Oxalis*   In this connection it seemed desirable to examine the data in the literature   There are very few analyses of plant saps sufficiently complete for our requirements and these deal chiefly with cells free from chlorophyll, which are not very useful for our present purpose [1]   But there are numerous cases in which leaves or entire plants were ashed and analyzed (these will be referred to as analyses of total ash)

A selection of the published leaf ash analyses has been treated in such a way as to show the proportion of cation to anion equivalents, (*a*) in the total ash, and (*b*) in the cell sap   In arriving at the latter values (Column 15, Table I, p 284) the probable composition of the sap has been calculated by means of certain corrections applied to the total ash analyses, according to the scheme outlined below [2]

It was recognized that to calculate the composition of the sap deductions should be made from the data for total ash to account for the following

(*a*) Polar substances precipitated in the cells (*e g* calcium oxalate, carbonate, pectate, and silica)

(*b*) Substances which yield on ashing inorganic substances, origi-

---

[1] See for example, the analyses of orange juice lemon juice etc. in the literature.

[2] Fe_2O_3 and SiO_2 are usually reported in ash analyses but since little is known of their mode of entrance into the plant except that they are probably colloidally dispersed they have been omitted from both total ash and sap analyses

TABLE I

Analyses of Total Ash of Leaves*

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
	Ash as per cent of dry weight	Equivalents per 100 gm of ash								Total cation equivalents in total ash	Total cation equivalents in sap	Total anion equivalents	Ratio Cation equivalents / Anion equivalents	
Plant		K+	Na+	½ Ca++	½ Mg++	½ Mg++ corrected	½ HPO4=	½ SO4	Cl				In total ash	Calculated for sap
1 Rumex acetosella	8 14	0 6766	Trace	0 7357	0 3168	0 2891	0 3094	0 2450	0 1268	1 7291	0 9657	0 6812	2 54	1 42
2 Rheum rhaponticum	7 93	0 3079	0 0250	0 1411	0 2767	0 2483	0 3096	0 2380	Trace	1 7507	1 5812	1 0940	1 60	1 45
3 Trifolium pratense	8 11	0 6964	0 1832	0 2330	0 3792	0 3515	0 8560	0 4900	0 1566	2 4918	1 2311	0 4182	5 95	2 95
4 Quercus robur	3 50	0 7051	Trace	0 9318	0 2450	0 2483	0 2126	0 0490	0 0034	2 3067	1 3106	0 4200	5 56	3 16
5 Arum maculatum	8 35	0 4321	0 3381	1 0001	0 1840	0 6055	0 4849	0 2140	0 0465	2 5271	1 2651	0 4559	6 02	3 02
6 Morus alba	7 18	0 6653	0 0130	0 1950	0 3698	0 3560	0 5878	0 3382	0 0017	2 5671	1 3531	0 5710	5 63	2 97
7 Spinacia oleracea	16 27	0 4985	0 0723	0 5457	0 1441	0 1210	0 2408	0 1160	0 0017	2 0763	1 8675	0 3290	3 64	3 27
8 Avena sativa	9 71	0 9336	0 0723	—	2 0060	0 2564	0 2068	0 1052	0 0753	2 0192	1 1269	0 2174	5 15	3 42
9 Olea europea	4 54	0 5589	—	2 0060	0 1760	0 8975	—	0 1606	0 1830	1 6957	0 7657	0 6239	12 98	3 52
10 Vicia sativa	—	1 1010	0 2987	1 5729	0 7393	0 4767	0 4113	0 3644	0 0195	2 2803	2 2972	0 4509	7 17	3 68
11 Syringa vulgaris	3 47	0 6751	0 2226	0 5000	0 4872	0 7901	0 0619	0 1970	0 0725	2 0670	1 6593	0 5456	5 46	3 68
12 Stellaria media	13 18	0 7486	0 7486	0 3700	0 4872	0 4194	0 4345	0 1306	0 2082	2 2761	2 0331	0 4343	4 67	3 73
13 Beta vulgaris	15 09	0 8570	0 17811	0 8836	0 6540	0 6267	0 1598	0 2082	0 0643	2 5502	2 6615	0 5456	5 456	3 83
14 Ficus carica	8 26	0 3166	0 0394	0 3104	0 3550	0 3104	0 6267	0 2168	0 1407	2 0955	2 1636	1 2144	4 98	4 13
15 Medicago sativa	8 71	0 9917	0 0394	0 1931	0 5100	0 1931	0 3079	0 0405	0 1760	2 5047	2 5047	2 3904	9 21	2 12
16 Daucus carota	13 61	0 2062	0 2062	0 1599	0 0140	0 1599	0 1765	0 2528	0 2250	2 1251	1 2030	0 5934	3 64	2 03
17 Cichorium intybus	12 46	0 2750	0 0248	0 1599	0 16812	0 1599	0 1417	0 0920	0 1170	2 2268	2 5746	0 4115	4 34	2 71
18 Citrus aurantium	10 53	0 3513	0 2750	0 2832	0 5482	0 2618	0 2832	0 2575	1 1482	2 3769	1 9697	0 7812	5 934	2 42
19 Atropa belladonna	10 81	0 6728	0 6728	0 3203	0 9203	0 1931	0 3203	0 2210	1 2250	1 9697	2 8166	1 5351	8 70	2 45
20 Primula farinosa	11 73	0 4289	0 2813	0 6104	0 2813	0 6104	0 5913	0 1254	1 1477	2 3103	2 2409	1 3015	3 84	2 23

* Except No 1 for which see p 293
† For English names see footnote 43, p 292

nally present in non polar linkages (e g chlorophyll yielding MgO and proteins yielding SO_3)

(c) Substances insoluble in water but dissolved in the non aqueous part of the protoplasm which yield on ashing inorganic oxides (e g lecithin yielding P_2O_5, calcium diglyceryl phosphate yielding both P_2O_5 and CaO)

(d) Inorganic anions and cations in combination with proteins in the protoplasm   But any proteinates in the protoplasm must have been formed by the interaction of MOH with the acid part of the protein and this is practically equivalent to the entrance of MOH so no deduction will be made (M is any inorganic cation)

(e) Inorganic anions and cations in solution in the intercellular liquid

(f) Inorganic ionogenic substances in the protoplasm

There is unfortunately no way of estimating these last two corrections   But it may be assumed that the amounts involved are small compared with the amounts of inorganic substances in the cell sap

We shall now consider each of the ash constituents in turn in order to determine approximately what corrections should be applied

Sodium and Potassium —The idea that the alkali metals can be held inside cells in non polar linkages seems to be fairly prevalent [3]   But the recent investigations of Weevers,[4] Penston,[5] Lloyd [6] Maquenne and Demoussy,[7] Canals

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[3] Mitchell P H , General physiology, New York McGraw Hill Book Company 1923 88   Guillemin M , and Larson, W P *J Infect Dis* , 1922 31, 349   Camlong S and Genevois, L *Bull Stat biol Arcachon*, 1930 27, 209   Macallum A. B *J Physiol* 1904–05, 32, 93   Stoklasa, J *Biochem Z* Berlin 1917 82, 310   *Z landw Versuchswesen Österreich* 1908 11, 52   Bialascewicz, K , quoted from Needham J , Chemical embryology Cambridge University Press 1931 1, 363   Inozemtzev (Inozemtzev, S I , *Ergebn Veg Lab Vers Prjanish nikov* 1930 16, 85) has reported that when the plant sap is electrodialyzed up to 84 per cent of the total potassium in mature plants cannot be dialyzed and of the dialyzable portion an appreciable part goes to the anode   He argues from this that most of the potassium must be completely bound in an organic compound

[4] Weevers T , *Rec. trav bot néerl* 1911 8, 289

[5] Penston (Penston N L   1m *J Bot* 1931 46, 673) states that all the potassium of potato tissues is in the cytoplasm or vacuole.

[6] Lloyd, F E *Flora* 1925 118–119, 309   Lloyd, F E , and Moravek, V *Plant Physiol* , 1928 3, 101

[7] Maquenne L and Demoussy E *Compt rend Acad* 1914, 158, 1400

Canaye, and Cabanes,[8] Kostytschew and Eliasberg,[9] and Hill and Kupalov[10] indicate that this is not the case[11]   This conclusion seems probable on chemical grounds, for it is clear that according to the modern theory of valence it would be very difficult to form compounds of sodium and potassium (second and third members of the a subgroup of Group 1) in which the metal is covalently instead of electrovalently bound[12]   The organic compounds of these elements which have been investigated are salt-like in character (with one or two possible exceptions)   Those in which the metal is not directly linked to a carbon atom, such as salts of organic acids, are in general soluble and are hydrolyzed more or less according to the strength of the acid   Those in which the metal is apparently joined to a carbon atom are such as the alkali alkyls (e g $NaCH_3$), the alkali aryls (e g NaC_6H), the alkali alkyl aryls (e g $\begin{matrix} C_6H_5\searrow \\ C_5H_5\!\!-\!\!CNa \\ C_6H_5\nearrow \end{matrix}$), are extremely unstable, some of them uniting explosively with oxygen when exposed to the air and all of them being decomposed violently by a trace of moisture   They are also salt-like in organic solvents   It was formerly supposed by Schlenk and Holtz[13] that the colorless compounds of this group were non-polar but it has since been established by Hein and his coworkers[14] that like colored members they are ionized in suitable solvents   For example, sodium ethyl and other colorless compounds of this class conduct electrolytically in zinc ethyl which is itself a non-conductor   Accordingly, as Rodebush[15] points out, if we could liquefy a compound such as sodium ethyl without decomposition we have every reason to suppose that it would show the ionization and conductance of a fused polar salt   Other groups of metal to carbon linkage compounds are formed by the addition of the metal to unsaturated groups, such as $-C \equiv C-$ and $-C \equiv N-$ and $-C \rightleftharpoons O-$, and by the addition of alkali alkyls and aryls to $-C \equiv C-$ Although the physical chemistry of these compounds has not been investigated

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[8] Canals, L , Canaye, J , and Cabanes, E , *Bull Soc chim biol* , 1930, **12**, 1022

[9] Kostytschew, S , and Eliasberg, P , *Z physiol Chem* , 1920, **111**, 228

[10] Hill, A V , and Kupalov, P S , *Proc Roy Soc London, Series B*, 1930, **106**, 445

[11] See also Willstatter and Stoll (Willstatter, R , and Stoll, A , Untersuchungen über Chlorophyll, Berlin, Julius Springer, 1913) who nowhere find that native chlorophyll contains potassium

[12] Fajans, K , *Naturwissenschaften*, 1923, **11**, 165

[13] Schlenk, W , and Holtz, J , *Ber chem Ges* , 1917, **50**, 262

[14] Hein, F , *Z Elektrochem* , 1922, **28**, 469   Hein, F , Petzchner, E , Wagler, K , and Segitz, F A , *Z anorg u allg Chem* , 1924, **141**, 161   Hein, F , and Segitz, F A , *Z anorg u allg Chem* , 1926, **158**, 153

[15] Rodebush, W H , *Clem Rev* , 1928, **5**, 526

carefully it may be assumed that they also are polar In any case they are decomposed by water very readily [16]

This suggests that the possibility of the occurrence of non polar compounds of sodium and potassium in plant tissues is remote, as indeed Kostytschew and Eliasberg[9] concluded on experimental grounds

It is possible also that a part of the alkalies appearing in the ash may be derived (a) from insoluble salts This is unlikely because of the general solubility of alkali salts in water (b) From salts dissolved in the oily part of the protoplasm In this connection it has been suggested in a former paper[17] that HA, the substance in the protoplasm, which furnishes anions to transfer cations between the outside and the vacuole may be a diglyceryl phosphoric acid Hundeshagen[18] has shown that the alkali salts of such compounds are more soluble in some non polar solvents than in water and Chibnall and his coworkers[19] have found similar substances in the protoplasm of a variety of cabbage (*Brassica oleracea*, L) and crow s foot grass (*Dactylis glomerata*, L) in the form of the calcium salts It is of course, quite improbable that any of the purely inorganic salts of sodium or potassium will be as soluble in the oily part of the protoplasm

Probably also in some leaves a certain amount of alkali proteinates is dispersed in the protoplasm In others the protein is cationic.

---

[16] Some recent work by Sidgwick and Brewer (Sidgwick N V, and Brewer Γ M *J Chem Soc*, 1925, **127**, 2379) suggests that organic compounds in which sodium or potassium are covalently linked in chelate rings are possible These compounds are the result of the reaction between the metal hydroxide and beta diketones and similar compounds Usually the product is salt like in character, but in a few cases derivatives have been obtained which are non polar since they dissolve in toluene and other non hydroxyl organic solvents and have definite melting points The authors assign the following structure to the benzoyl acetone derivative
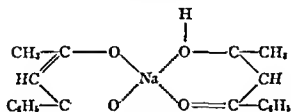
in which the metal is bound covalently between two chelate rings. These substances are very unstable in the presence of water

[17] Jacques, A G, and Osterhout, W J V, *J Gen Physiol*, 1933-34 **17**, 727

[18] Hundeshagen, Γ, *J prakt Chem* 1883 **28**, N s , 219

[19] Smith J A B and Chibnall, A C, *Biochem J* London 1932 **26**, 1345 Chibnall, A C, and Channon, N J *Biochem J*, London, 1929, **23**, 176

Since alkali and alkali proteinate are formed by the interaction of alkali hydroxide with an acid group, no correction will be applied to the ash analyses to account for them   In any case the amount of alkali combined with X is certainly small and the calculation below indicates that the correction for alkali proteinate does not exceed 5 per cent   According to Chibnall and Grover[20] the proteins extracted from leaves are similar to glutelins but as far as we know the predominant plant proteins are globulins   According to Kodama[21] one of these, edestin, has a combining weight of 5402 at pH 9 34   The pH outside the protoplasm may possibly reach this value due to photosynthesis   Svedberg[22] has found that the molecular weight of edestin is 208,000   Green,[23] on the basis of the Debye-Huckel theory, has calculated that its "valence type" around pH 7 0 is about 25, which makes it either *quinque-quinque* or 25 valent   The latter figure gives a combining weight 8320   The true combining weight no doubt lies between this figure and 5402   But taking 5402, then $\dfrac{75}{5402}$ = 0 014 equivalent of alkali cations might conceivably be present in the protoplasm in combination with protein   But Chibnall considers that the amounts of protein he was able to extract from leaves are less than the actual amounts present, due to losses of various kinds, so that it may be assumed that 150 gm of protein are present in 1000 gm of dry leaves   This amount could hold in combination 0 028 equivalent of alkali cations or roughly <3 − 5 per cent of the total alkali present   This calculation ignores the fact that at least some Ca and Mg proteinates must also be present

Magnesium —Magnesium appears to be deposited in plant tissue as phosphate, but this is so rare as to be negligible   Some magnesium may also be bound by protein, but here again, magnesium proteinate is formed by the interaction of $Mg(OH)_2$ and the acid portion of the protein molecule and no correction has been applied   The only other important non-sap occurrence of magnesium is in the chlorophyll   In the case of green leaves a correction has been computed for each case on the basis of the average amount of chlorophyll in green leaves as given in the literature   Thus Willstatter and Stoll[24] found that for the leaves of five common trees, the average chlorophyll content was 0 8 per cent of the fresh leaves or 2 5 per cent of the dry leaves   Similar values have been found by Lubimenko[25] and by Sjoberg[26]

[20] Chibnall, A C , and Grover, C E , *Biochem J* , London, 1926, 20, 108

[21] Kodama, K , *J Biol Chem* , Japan, 1922, 1, 419

[22] Svedberg, T , *J phys et radium*, 1931, 2, 227

[23] Green, A , *J Biol Chem* , 1932, 96, 47

[24] Willstatter, R , and Stoll, A , Untersuchungen uber Chlorophyll, Berlin, Julius Springer, 1913, 112

[25] Lubimenko, A Compt rend Acad , 1924, 179, 1073

[26] Sjoberg K , *Biochem Z* , Berlin, 1931, 240, 156

In general the asb is about 10 per cent of the dry weight of the leaves so that 100 gm of asb which corresponds to 1000 gm of dry leaves would contain the magnesium derived from 10 gm of chlorophyll But chlorophyll a or b contains about 2 7 per cent Mg which corresponds to 0 27 gm of Mg or 0 0225 equivalent of Mg in 100 gm of ash

Apparently therefore a small but not negligible correction ought to be applied to the magnesium asb results for green leaves and this has been done by assuming in each case that the chlorophyll content is 1 per cent of the dry leaf weight of the leaves and calculating from the published data the weight of leaves necessary to produce 100 gm of ash For example the pure nsb of *Beta vulgaris* is given ns 15 09 per cent in Wolff, nnd hence 669 gm of dry leaf correspond in this case to 100 gm asb, and the Mg correction would be 0 0149 equivalent

Calcium —Calcium occurs in polar but insoluble form, as the oxalate, sulfate, nnd phosphate in plant tissues and according to Chibnall and Channon[27] ns Ca salts of diglyceryl phosphoric acid, dissolved in the cytoplasm. The results of Chibnall and Channon indicate in the case of cabbage leaf that about 2/3 of the Ca present is water soluble   Kostychev and Berg [8] find for a series of plants only about 1/3 of the Ca to be extracted by water the remainder being present in insoluble ionogenic form   Calcium may also be present in combination with protein as a proteinate and possibly also combined in a non polar manner

In our calculation to sap composition, in order to avoid the inclusion of calcium not originally present as ions we have corrected the asb data by excluding Ca entirely

Iron —There is very little evidence for the assumption that iron is present ns a cation in leaf sap, although the asb yields appreciable amounts of Fe_2O_3 How ever, Jones[29] bas suggested recently on the basis of microprecipitation reactions that ionogenic iron is widespread in vegetable tissues   This is contrary to the work of Chibnall and Channon who found no ' soluble iron in cabbage leaf nnd of Maquenne and Cerighelli[30] who found that the expressed and centrifugalized juices of romaine, lettuce and other vegetables high in iron content contained very little iron   On the basis of these latter results we have corrected the ash data for iron by omitting it from the cations

Chloride —As far ns we can determine no compounds have been isolated in plants in which chlorine was either present in an insoluble polar compound or coordinately bound in nn organic molecule   Jung[31] who bas examined numerous

[27] Chibnall, A C and Channon, H J , *Biochem J* London 1929, 23, 176

[8] Kostychev S and Berg V *Planta*, 1929 8, 56

[9] Jones H W , *Biochem J* London 1920 14, 654

[30] Maquenne, L , and Cerighelli R *Bull Soc chim France*, 1921 29, series 4 899

[31] Jung J , *Sitzungsber K Akad Wissensch Math naturwissensch Cl Wien*, 1920 129, 297

plant tissues concludes that the chlorine is always present in plants as chloride He found most of the chloride in the leaves

Wood[32] found that in the xerophytic plants *Atriplex* and *Kochia*, which take up a great deal of NaCl from the soil, the amount taken up by the leaves was roughly a function of the amount in the soil, and that most of it was in the veins, not in the cells   It is well known that seashore plants also take up sodium chloride in large quantities, and Wood's work at once suggests that where the chloride content is abnormally high, particularly when the sodium content is also high (which seems to be nearly always the case), a great part of it is present as sodium chloride in the veins, and should not be counted as cell sap   It is not possible to correct for this condition quantitatively   But it is a point to be borne in mind wherever the chloride and sodium contents are unusually high   Obviously the presence of much NaCl in the leaf veins will lower the ratio cations − anions

Sulfate —Brunswik[33] found that crystals of calcium sulfate were abundant in the leaves and young stems of the Tamaricaceae, but generally, sulfate is not present in crystals precipitated in the cells   A certain amount of sulfur is present in some cases in the form of essential oils, but as these are volatile not much sulfate ion will find its way into the ash from this source   Probably in leaves the chief source of sulfate ion which was not originally present as such in the cell sap is the sulfur coordinately bound up in the protein of the protoplasm and the sap   Assuming, as before, that there are 150 gm of protein present for each 100 gm of ash, if the sulfur content is taken as 1 per cent, the sulfate which might be derived from this source would be 0 1 equivalent   However, as Bertrand and Silberstein[34] have shown, up to 50 per cent of the sulfur present in the plant is lost when the plant is ashed instead of being oxidized by wet methods, so that the deduction on this account might be 0 05 equivalent   However, in order to avoid overcorrection of an anion no correction has been applied

Phosphate —In some plants calcium or magnesium phosphate crystals occur, but this is rare

The chief source of phosphoric acid in leaves is probably the phospholipins (phosphatides) or related substances of the protoplasm, such as the Ca diglyceryl phosphatidate found by Chibnall and Channon in the leaf protoplasm of *Brassica oleracea*   Chibnall and Channon found that about 75 per cent of the total phosphate of the leaf is water-soluble, but their ether-soluble phosphoric acid derivatives accounted for only about 10 per cent of the remainder   André[35] has treated dried and powdered lilac and chestnut leaves with alcohol and ether, to extract what he calls the organic phosphorus   It appears from his results that about 10 per cent of the total phosphorus, according to this criterion, is organic

[32] Wood, J G , *Australian J Exp Biol and Med Sc* , 1925, **2**, 45

[33] Brunswik, H , *Sitzungsber K Akad Wissensch Math -naturwissensch Cl* , II er 1920, **129**, 115

[34] Bertrand, G , and Silberstein, L , *Compt rend Acad* , 1929, **189**, 886

[35] André, G , *Compt rend Acad* , 1916, **162**, 563

In addition to the well known phosphatides and derivatives, such as Chibnall found in leaves and which are known to be present in larger quantities in seeds, Hansteen Cranner, Grafe and Magistris[36] have claimed to have extracted by water alone phosphatides from living plant tissues such as carrots, beets, etc., without injuring the tissue But this is disputed by Steward [37]

On the whole it appears that about 80 to 90 per cent of the phosphate in the ash was originally present in the cell sap as such and in order to avoid any suspicion of over correction we shall make no deduction from the published phosphate figures However, we shall regard the phosphoric acid as a dibasic and not a tribasic acid because the pH of plant saps is almost always below 7 0 where the PO_4^{---} ion can exist only in infinitesimal amounts

Silica —Silica is almost always present as deposited SiO_2 but it is clear that in the intracellular liquid it must be either dissolved or colloidally dispersed, since it makes its way from the roots and deposits in part in the leaves As long ago as 1878 Lange[38] concluded that it is present as colloidally dispersed free silicic acid, and this conclusion is accepted by Nanji and Shaw[39] in their work on silica in straw These writers, however, believe that about 10 per cent of the silica might be present in the form of carbohydrate esters but no such compounds were isolated

In any case it may be assumed with confidence that there are no silicate ions in the cell sap, since as Hagg[40] has recently shown the dissociation constants of H_2SiO_3 which he considers to be the only true silicic acid are of the order of 10^{-9} and 10^{-13}

Accordingly we shall assume that the silica of the ash analyses contributed no anions to the cell sap

Nitrate —It is conceivable that there is present in the sap nitrate ion This does not appear in the ash analyses It is difficult to estimate what correction should be made but it may be concluded that it is small Thus according to Campbell s results[41] for twenty five weeds the nitrate nitrogen did not exceed 0 80 per cent of the dry weight of the plant tissue In most cases it was much less and at maturity it was almost always absent

There are present also basic nitrogen compounds such as ammonia and organic bases which are cationic at the C_R encountered in leaf saps These also disappear in ashing so that the error due to the failure to account for anionic nitrogen is offset to some extent

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[36] Magistris H , *Ergebn Physiol* 1931, 31, 165 (an account of the work on phosphatides with an extensive bibliography)

[37] Steward F C , *Brit J Exp Biol* , 1928–29 6, 32 *Biochem J* , London, 1928 22, 268

[38] Lange W *Ber chem Ges* 1878 11, 822

[39] Nanji, D R and Shaw W S *J Soc Chem Ind* London, 1925 44, 1T

[40] Hägg G *Z anorg u allg Chem* , 1926 155, 21

[41] Campbell E G *Bot Gaz* , 1924, 78, 103

Ionogenic Inorganic Substances in General —The processes of drying and ashing employed in reducing plant tissue to an analyzable form are drastic   Thus various sulfur compounds are probably oxidized to SO_3 or H_2SO_4 Phosphoric acid is freed from the organic combinations in which it is usually found in plant materials, and probably certain amounts of pyrophosphoric and metaphosphoric acids are produced   All these acids because of their lower volatility are capable of decomposing chlorides, so that if any appreciable amounts of free acids are formed chloride ion may be replaced   This is an additional reason for not making a correction for sulfate or phosphate

Further, in the course of drying the plant material there is the possibility that magnesium chloride and possibly calcium chloride may be hydrolyzed in part to basic chlorides with a corresponding loss of chloride ion   However, since nearly all the water is driven off in the neighborhood of 100°C this loss is probably not serious, for as a number of investigators have shown the hydrolytic decomposition of hydrated magnesium chloride is not marked below 200°C in air [42] Hydrated calcium chloride is even more stable

Summarizing, we conclude that to transform ash analyses of total ash into analyses of sap the following corrections should be made Ferric or ferrous iron and silicate should be omitted since the amounts dissolved in the cell sap are negligible   Calcium, although some may be dissolved in the sap, should be omitted since the amount is doubtful A deduction should be made in the case of magnesium to allow for the amount contained in chlorophyll   No deduction should be made from the sulfate or phosphate figures, but phosphate should be taken as bivalent   No correction should be applied to sodium, potassium, or chloride

Table I[43] (p 284) gives figures derived from the data in the literature   In the original these results were expressed as percentages of

[42] For references see Mellor, J W , A comprehensive treatise of inorganic and theoretical chemistry, London, Longmans, Green and Co , 1923, 4, 300

[43] The data from which the figures in Table I are derived were taken from Wolff, E , Aschen-Analysen von landwirtschaftlichen Producten, Berlin, Wiegandt und Hempel, 1871   All relate to leaves except the figures for *Rumex acetosella* which apply to the whole plant in which the volume of root and stem is small as compared to that of the leaf (as explained on p 293 the whole plant may be included without error)   The English names are as follows (1) field sorrel, (2) rhubarb, (3) red clover, (4) oak, (5) wake robbin, (6) mulberry, (7) spinach, (8) oat, (9) olive, (10) tobacco, (11) lilac, (12) chickweed, (13) beet, (14) fig, (15) alfalfa, (16) carrot, (17) chicory, (18) orange, (19) deadly nightshade, (20) primrose

oxides in the pure ash   Here they are expressed as equivalents of the corresponding basic or acidic radical per 100 gm of pure ash   They are therefore not conventional concentration terms   These cannot be estimated

Let us first consider the total ash   Columns 1 to 6, and 8 to 10 are self explanatory   Column 11 is the sum of Columns 3, 4, 5, and 6   Column 13 is the sum of Columns 8, 9, and 10   Column 14 is obtained by dividing the values in Column 11 by those in Column 13

Let us now consider the composition of the sap as calculated from the total ash by the method summarized on p 292   Column 7 gives the values for magnesium after deducting what was probably present in the chlorophyll   Column 12 is the sum of Columns 3, 4, and 7   Column 15 is obtained by dividing the values in Column 12 by those in Column 13   To the extent that the corrections are right, Column 15 gives the actual preponderance of inorganic cations over anions in the sap   These results are comparable therefore with those given in Part 1, for the leaf saps of *Rheum*, *Rumex*, and *Oxalis*   The selection of data yields ratios of cations/anions both higher and lower than the average 3 8 given in the table on p 239 of Part 1   For the most part plants showing a fairly high ratio of cations to anions in the sap were selected for Table I as being of more interest from our present point of view, but it should be pointed out that in only a very few of the hundred or more cases examined in the literature were the anion equivalents greater than cation equivalents in the corrected figures for sap, and in no case was this true of the total ash

The above analyses are of leaves only (except *Rumex acetosella*[44])   When organic substances manufactured in a green cell migrate to a colorless cell of the same plant and are analyzed along with the green cells they may be regarded as on practically the same basis as if they still remained in the green cell   Hence the colorless cells of the leaf, or of the root and stem of the same plant, may be included in the analysis along with the green cells

It is, however, misleading to take analyses of colorless cells by them selves (e g fruits, roots, fungi, etc)   since these cells may have ab sorbed inorganic cations paired with organic anions   Usually a high ratio of cations to anions is found in such cells (in the banana[44] fruit,

[44] Czapek F, Biochemie der Pflanzen, Jena, Gustav Fischer 3rd edition 1922–25, 2, 464

Musa sapientum, the ratio of cation to anion equivalents in the total ash is 8 64)

In this connection we may consider the situation in animal cells Hill and Kupalov,[10] and likewise Fenn[45] and Cobb, state that there is an excess of inorganic cation equivalents in frog muscle   Page[46] found this to be true of eggs of the sea urchin (*Arbacia*) and of the starfish (*Asterias*) which are devoid of yolk   It also applies to the white of hen's egg but it is evident from Needham's[47] summary that in the yolk there is so much sulfur and phosphorus in organic combination (yielding inorganic anions on ashing) that the anion equivalents preponderate   As the yolk is used up in development the situation changes and the cation equivalents predominate more and more   It is of interest to note that such a predominance is also found in the human embryo [48]

DISCUSSION

As already stated (Part 1, p 235) our experiments on *Valonia* were thought to indicate that the cations enter chiefly as MOH and react in the sap with an organic acid, HA, to form KA This would mean that the penetration of cations is necessarily accompanied by the formation of organic salts which should be capable of detection when they exist in sufficient quantity

In the experiments described in Part 1 of this paper such salts were found and their presence in other cases (Part 2) is shown by the fact that the inorganic cation equivalents exceed the anion equivalents As already mentioned this excess has been expressed in two ways in Table I as the ratio of cations to anions in the total ash, and as the ratio of cations to anions in the cell sap   The sap data are chiefly interesting from the standpoint of accumulation where the relation between the soluble electrolytes of the sap and the external solution

[45] Fenn, W C , in Physical and chemical changes in nerve during activity, Occasional publications of the American Association for the Advancement of Science, No 2, *Science*, 1934, 79, suppl

[46] Page I H , *Biol Bull* , 1927, 52, 168

[47] Needham, J , Chemical embryology, Cambridge University Press, 1931, 1 356 3, 1276

[48] Needham, J , Chemical embryology, Cambridge University Press, 1931, 3, 1276

is important   At present, however, we are concerned chiefly with the question whether an excess of inorganic cations over inorganic anions has entered the leaf cells   From this point of view all the inorganic constituents (except iron and silica which for reasons mentioned before are excluded) should be counted, because, regardless of the state in which they happen to be in the leaf cells at the time of analysis, obviously at some stage in the plant's growth they have entered[49] as part of a dissolved electrovalent compound   (For example, many green cells contain calcium oxalate crystals, and this calcium, not in solution in the cell sap, must be counted, since there is no reason to suspect that its mode of entrance is different from that of calcium which may be present in another cell as dissolved calcium chloride)   The same is true of the anions which form inorganic elements such as sulfur and phosphorus, and hence all sulfate and phosphate reported in the ash should be counted   However, it must be admitted that the anion total is probably too low because, as Bertrand and Silberstein[31] have shown, in the dry ashing up to 50 per cent of the sulfur is lost   Further, as pointed out below, some of the SO_4^{--} and HPO_4^{--} present may represent chloride which has been displaced during ashing

Hence in determining whether cations have entered as hydroxides we should use the figures for total ash

Before concluding that excess of cation equivalents means the entrance of cations combined with hydroxyl we must examine other alternatives   We may now consider the various possibilities in turn

(a) *Ionic Exchange*—When the external solution loses a molecule of MOH it is equivalent to losing M^+ and gaining H^+ and when MOH reacts in the sap with HA to form MA it is equivalent to losing H^+ from the sap and gaining M^+ Hence the net result is the same as if H^+ from the sap were exchanged for M^+ from the external solution, the ions passing as such through the protoplasmic surface

For reasons elsewhere[50] set forth in detail it seems improbable that a

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[49] 'Entrance in this connection means simply passage from the aqueous external solution through the outer non aqueous protoplasmic surface   It does not imply penetration as far as the vacuole although this usually occurs   However a certain amount of magnesium passes into the protoplasm and is combined in the chlorophyll molecule   This is counted as having entered the cell

[50] Osterhout W J V   *Ergebn Physiol* 1933 35 967   Jacques A. G   and Osterhout, W J V , *J Gen Physiol* 1933–34 17, 727

passage of ions as such through the protoplasmic surface plays an important part in the penetration of electrolytes and hence in the subsequent discussion it will be regarded as a negligible factor

(b) *Entrance of Organic Electrolytes* —In the case of cells devoid of chlorophyll, whether plant or animal, the excess of cation equivalents might be thought to indicate the entrance of cations in combination with organic anions   But this cannot be the case with cells containing chlorophyll which manufacture organic from inorganic materials and can at best absorb only minute amounts of organic substances from the soil

(c) *Entrance of Nitrates* —If a cation should pass into the protoplasm in combination with nitrate the latter might be reduced and built up into the protein molecule, leaving the cation free to pair with an organic anion elaborated in the vacuole   The ash would then show an excess of inorganic cations over anions   To what extent this process can occur is as yet unsettled   Certainly nitrate ion enters the plant and is used in protein synthesis   (This entrance could occur by exchange, in the same way as we believe that Cl^- is exchanged for HCO_3^- in *Valonia*)   The amount of nitrate ion present in leaves is seldom more than a trace, and in many cases it is entirely absent Thus in the sap extracted from *Rheum, Rumex,* and *Oxalis* we found no definite reaction for nitrate ion   (This, of course, does not mean that no nitrates were taken up by the roots for it is well known that such nitrates may not appear in the leaves[51] presumably because they are reduced in the root or stem to ammonia which enters the leaf cells as such, or as NH_4OH, or as some other compound)   However, if this ion goes into the protein molecule, we should scarcely expect to find more than a trace

If it may be assumed that all the protein of leaves has been derived from MNO_3, it is possible to estimate the maximum amount of M

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[51] Prianischnikow, D N , *Ergebn Biol* , 1926, 1, 427, 432   For the literature see also, Tiedjens, V A , and Robbins, W R , *New Jersey Agric Exp Station, Bull No 526 1931   Hoagland, D R , Annual review of biochemistry, Stanford University, Stanford University Press, 1932, 1   According to Vickery, H B , Pucher, G W , Wakeman, A J , and Leavenworth, C S , *Carnegie Institution of Washington, Pub No 445*, 1933, the nitrates found in dried leaves may have been formed, in part at least, during the process of drying

(cation) which may have entered the leaf cells in this manner   Thus, as pointed out previously, according to the results of Chibnall and Grover[20] there appears to he on the average 150 gm of protein per 100 gm of pure ash in leaves   If 16 per cent of this is nitrogen, this corresponds to $\dfrac{150 \times 16}{14 \times 100} = 1\ 7$ equivalents of nitrate nitrogen   As Tahle I shows this is usually greater than the cation total in the sap   Further the weight of evidence[52] suggests that tbe primary products required for the synthesis of proteins (the amino acids) are produced in tbe leaf and are transferred to other parts of the plant for the com pletion of the synthesis   On this hasis it is clear that far more than enough nitrate could enter the leaf to account for all the cations which have entered

But the experiments of Prianischnikow[51] and others indicate that ammonia is taken up in preference to nitrates so that when NH_4NO_3 is present the external solution becomes acid

It seems possible that in the leaf cells studied by us some of the nitrogen was taken up in the form of NH_3 and NH_4 as well as in the form of NO_3 In this case the ratio of cations to anions might not be greatly affected [53]

But there is no reason to suppose that if potassium entered as nitrate the rate would be affected by changes of pH as was found in experi ments on *Valonia*   Here it seems very improbable that the entrance of nitrates plays an important rôle   Moreover unless there is a con siderahle loss of nitrogen (which is highly improbable) the cell as a wbole would contain at least as many equivalents of protein nitrogen as of potassium if the latter entered solely as nitrate   this is certainly not tbe case in *Valonia*

(d) *Entrance of Bicarbonates* —If the cations entered in combina tion with bicarbonate ion, and if CO derived from the latter were subse quently used in tbe pbotosynthesis of carbohydrates, the cations could pair with organic anions in the cell   This situation, of course, would

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[52] See Onslow M W Principles of plant biochemistry, Cambridge University Press, 1931 chapter V

[53] Presumably cations would be used up first since to make protein NO_3 must be converted to NH_3 this would lower the ratio of cation equivalents to anion equivalents

give an excess of inorganic cations on ashing   The proportions of bicarbonate ion to CO_2 on the one hand, and to carbonate ion on the other, depend, of course, on pH, but it happens that in sea water when the pH is changed from 8 to 9 the bicarbonate fraction of the system changes very little[54] so that we should expect that if the cations enter as bicarbonate little effect would be observed in the case of *Valonia*[55] on changing the sea water pH from 8 to 9   Actually, however, there was a more than three fold increase in the rate of potassium entrance when the pH was raised from 8 2 to 8 5 to 8 8

Other experiments on *Valonia*[56] show that CO_2 enters very rapidly with little or no penetration of bicarbonate   M M Brooks[57] states that the sap of cells exposed to bicarbonates became more alkaline (the pH being measured after the removal of CO_2 from the sap) and ascribes this to the entrance of bicarbonate but it is evident that the same result would be produced by the penetration of KOH

We therefore suppose that if the ionic activity product (H) (HCO_3) should at any time become greater outside equality would be promptly restored by the penetration of CO_2 rather than by that of bicarbonate   Since the cell is constantly producing CO_2 its movement is outward except when photosynthesis is taking place

It seems probable that such considerations also apply to the green cells of the leaf

(c) *Entrance of Sulfate and Phosphate* —Since both sulfur and phosphorus appear in organic combination in the protoplasm it is possible that cations may have entered in combination with sulfates or phosphates, which have subsequently been utilized in the protoplasm, leaving the cation free to pair with an organic anion   In the case of *Valonia*, however, this seems impossible, for the total amounts of organosulfur or organophosphorus compounds in the cell are certainly far too small to account for all the cation which has entered the cell

Summarizing then we believe that the above considerations show

[54] *Cf* Osterhout, W J V , and Dorcas, M J , *J Gen Physiol* , 1925–26, **9**, 255   There is little change in the concentration of HCO_3^- or CO_3^{--} when the pH changes from 8 to 9

[55] Jacques, A G , and Osterhout, W J V , *J Gen Physiol* , 1933–34, **17**, 727

[56] Jacques, A G , and Osterhout, W J V , *J Gen Physiol* , 1929–30, **13**, 695

[57] Brooks, M M , *Pub Health Rep* , *U S P H S* , 1923, **38**, 1470

that the suggestion that cations enter mainly in combination with hydroxyl ion is consistent with all the known facts of penetration in *Valonia* and also in green plant cells

Adopting this view our present picture of the entrance of cations is as follows MOH unites with a constituent of the protoplasm HX, as suggested by certain models,[58] forming MX which reacts in the sap with a weak organic acid HA to form MA and that A is then exchanged for Cl or other inorganic anions coming from the external solution

This process may be practically complete as in *Valonia*, or may be much less extensive as in the case of the leaves studied by us, and in those cited from the literature, which give a high ratio of cation to anion equivalents As intermediate cases we may mention *Chara ceratophylla*, Wallr, as described by Collander,[59] and *Nitella clavata*, as described by Hoagland and his associates,[60] where the ratio is about 1 1

SUMMARY

Analyses have been made of the inorganic constituents of the juices expressed from the leaves of *Rheum*, *Rumex*, and *Oxalis*

It has been shown that in all cases there is a large excess of inorganic cations over anions in the sap, the average ratio of cations to anions being 3 8 (Part 1, p 239)

The ash analyses of plant tissues (chiefly leaves) reported in the literature have been examined critically, and it has been shown that the preponderance of inorganic cations over inorganic anions in the ash and in the sap is general

It has been concluded that the excess of inorganic cations is consistent with the view that cations pass into the protoplasm chiefly in the form of hydroxides, and are accumulated either in the form of organic salts (such as the oxalates) or in non polar linkage

It has been concluded that practically all the potassium and sodium found in plant ash must have been present originally in the form of soluble ionogenic compounds, but that a considerable part of the cal

[58] Osterhout W J V and Stanley W M , *J Gen Physiol*, 1931–32 **15**, 667 Osterhout, W J V , *J Gen Physiol*, 1932–33 **16**, 529 Osterhout, W J V , Kamerling, S E and Stanley W M *J Gen Physiol* 1933–34, **17**, 445, 469

[59] Collander R *Acta bot fenn* 1930 **6**, 1

[60] Zscheile, Γ P Jr *Protoplasma* 1930 **11**, 481

cium and magnesium may have been present originally in the form of insoluble salts or as components of non-polar compounds

The methods whereby the cations, particularly potassium, may have been accumulated have been discussed, and it has been concluded that as it does not seem very probable that they enter chiefly as nitrates or bicarbonates we may suppose that they go in to a large extent as hydrates this is highly probable in the case which has been most carefully investigated (*Valonia*)

THE APPLICATION OF QUANTUM MECHANICS TO CERTAIN CASES OF HOMOGENEOUS CATALYSIS

II. Certain Aspects of Enzyme Action

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(A) In a previous paper (1) the catalytic effect of H^+ and OH^- ions on the hydrolytic splitting of the C—N linkage was investigated by means of quantum mechanical considerations. In this paper will be reported investigations as to the effect of foreign rigid dipoles on the energy of the activated configurations, and thus on the energy of activation. If the latter is lowered a positive catalytic effect is noted, and such a dipole might conceivably constitute the prosthetic group of an enzyme for the reaction in question.

The specific problems and method of attack may be briefly sketched as follows.

Given some configuration (we shall limit ourselves to the activated one) of the atoms involved in the reaction

$$(\equiv C-N\equiv) + (-OH) = (\equiv C-O-) + (\equiv N-H)$$
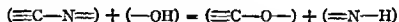
(i e under certain specific conditions this may represent the hydrolysis of a peptide link), and a separate dipole which may be made to assume and maintain any arbitrary position with respect to this configuration as diagrammed in Fig 1, then

1 What will be the nature of the effect of the dipole on the potential energy of the reacting system?

2 How close can the dipole approach the reacting configuration before the increase in potential energy of the six atom group (i e original four atom system plus the two atoms of the dipole) due to incipient interchange repulsions renders further approach impossible?

3 To what extent may such a dipole alter the activation energy of a reaction?

As examples of dipoles, arbitrary to be sure, we shall consider the C—O (of a carboxyl group for instance), the N=O of a nitro group and the N—H of an amino group, and study their effect in acid and alkaline solutions, since hydrolysis in solutions containing neither H nor OH ion is an idealized case

(B) The effect of the dipole AB on the energy of the reacting system can be best thought of as consisting of two factors   If it approach sufficiently near it will begin to form part of the reacting system   The bond A—B will begin to weaken and bonds A—N, A—H, A—C, A—O, B—C, etc , will begin to form and we have a six atom reaction

Dipole
Position a

Configuration of reacting
molecules
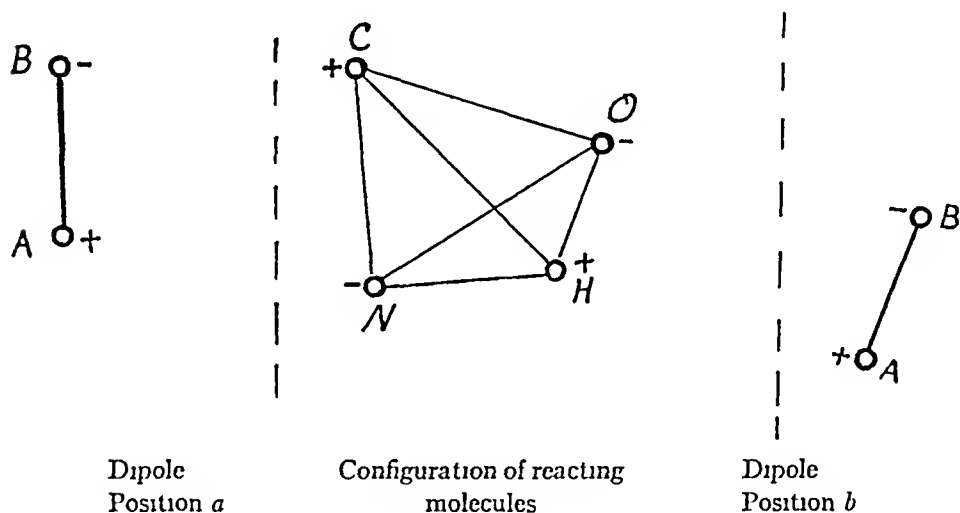
Dipole
Position b

Fig 1

In general the potential energy of the entire system will rise greatly On the other hand, if it remains at a sufficient distance its only effect will be an electrostatic or dipole effect on the four atom reacting system   This effect may either increase or decrease the potential energy of the four atom system   As drawn in Fig 1, Position a, the dipole would increase the potential energy of the reacting configuration This can be easily seen since there would be an attraction for the C—N group and a repulsion of the O—H group   This means that the reacting configuration of four atoms would be less stable than in the absence of the dipole, or, in other words, the O—H group would have to possess a greater amount of energy to approach the C—N to this position Such an effect would be a negative catalytic effect   However, were

the dipole AB rotated through an angle of 180° in the plane of the paper a different condition of affairs would result In such a case the group C—N would be repelled and the O—H group attracted The net result would be a decrease in the potential energy of the system and positive catalysis should occur The question remains as to the quantitative significance of the above pictured effects

(C) The first question which must be answered is how close the catalyzing dipole may approach the reacting system and still be treated separately and not as part of the reacting system This is done by studying the change in energy of the *six atom* system as the dipole is brought up from an infinite distance Details of calculation of this secondary problem are not given since it involves the setting up of a secular equation in the form of a symmetrical five row determinant, and the solution of this for the lowest root for every value of dipole distance Table I gives results of such calculations for the different dipoles approaching the reacting group as shown in Fig 1 but oriented in every case so as to give a positive catalytic effect It will be noted that the effect described is a maximum when the dipole is in the same plane as the reacting system Therefore the values of the dipole distance, r, are taken in the sense of distance from the nearer "edge" of the reacting system, and the dipoles are arbitrarily directed to have the line joining their constituent atoms parallel in every case with this nearer "edge "

From the last column of Table I it will be noted that, while the potential energy does not increase much up to an approach of about 3 Ångstrom units, the increase is very rapid beyond that and thus the actual approach will depend, among other things, upon the supply of energy available to overcome this repulsion [1]

[1] It is the purpose of the present paper to show that, given the dipole in proper position the activation energy of the four electron system will be lowered but not to investigate the cause of the dipole taking such a position against repulsive forces as given in Table I It is reserved for a future paper to investigate the possible nature of combination between enzyme and substrate from a quantum mechanical point of view The starting point will be suggested by the nature of the curves of Fig 4 of the previous paper (1), where especial emphasis will be laid on the basin or saddle in the middle of the curves If conditions were found such that the entire hill were sufficiently lowered but the basin retained, one might conceivably have the necessary energy for a practically unactivated loose combination between enzyme and substrate.

potential energy of the reacting configuration by the amount in the last column   This would result in a specifically increased reaction velocity of from 6- or 7- fold to some 4700-fold at room temperature, and in one case (the second figure) the reaction would be practically instantaneous unless some other configuration should become, in this instance, the activated one

(*E*) Northrop and Kunitz (4) have stated that "at present there is no direct evidence of the existence of any peculiar prosthetic group in these active proteins" (*i e* pepsin or trypsin) "not found in other proteins, and it is quite possible that their activity depends on some peculiar arrangement of the amino acids " The considerations presented in the present paper demonstrate, it is thought, that groups found in ordinary proteins may be capable of active catalysis if they can be properly placed with respect to the reacting system which is being catalyzed   Some of the necessary functions of the "peculiar arrangement of the amino acids" would, then, be to furnish, with the substrate, enough energy of combination to overcome the repulsive forces encountered by the dipoles in assuming an "active position," as well as to furnish such dipoles at the proper points   The latter named function would involve specific arrangements and is not amenable to general treatment, but the source of enzyme-substrate combination may be investigated and it is hoped in a final paper to indicate the possible source of such bonding

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SULFHYDRYL AND DISULFIDE GROUPS OF PROTEINS

I. METHODS OF ESTIMATION

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INTRODUCTION

There have been no methods for estimating quantitatively the sulfhydryl (SH) and disulfide (S S) groups of proteins. And yet enough is known of these groups to indicate their importance from several points of view. SH and S S groups appear in proteins during coagulation, and their appearance is the only change known to occur in the constitution of the protein on coagulation (Heffter, 1907, Arnold, 1911). They may be of interest for an understanding of the chemical changes which take place in muscle, SH groups can be detected in the proteins of minced muscle even before the tissue has been treated with a coagulating agent (Arnold, 1911). SH groups are also significant for the study of enzymes for in the case of crystalline urease it has been observed that activity depends upon the presence of these groups (Sumner, 1933).

In this paper methods for estimating protein SH and S S groups are described. It is shown for the first time that the number of SH and S S groups detectable in a coagulated but unhydrolyzed protein is equivalent to the quantity of cysteine and cystine found in the hydrolyzed protein. In native proteins, on the other hand, only a small fraction of these groups can be detected. To develop methods for estimating protein SH and S S groups we have used denatured proteins because the validity of the method can best be tested on such preparations. The methods are also applicable to native proteins, however, and the results obtained for various native proteins will soon be published.

We have developed two independent methods for estimating the reactive SH groups of unhydrolyzed protein   In the *direct* method the SH groups are oxidized by cystine, which is thereby reduced to cysteine, and the number of protein SH groups oxidized is estimated by measuring the amount of·cysteine formed   Cystine oxidizes all the SH groups and no other groups in the protein   In the *indirect* method SH groups are eliminated by treatment with an oxidizing agent or with iodoacetate, and the reagent added is removed   The protein is then hydrolyzed, its total cysteine content is estimated and compared with the cysteine content of untreated protein   The diminution in cysteine content is equal to the number of SH groups which reacted with the reagent   Instead of measuring the cysteine contents of hydrolysates one can estimate the SH groups of the unhydrolyzed treated and untreated protein by the reaction with cystine

The number of S-S groups in unhydrolyzed protein is estimated by the increase in number of SH groups caused by reducing the S-S groups to SH with thioglycollic acid

The cystine and cysteine contents of protein hydrolysates are estimated colorimetrically by their reactions with phosphotungstate Cysteine, but not cystine, gives a blue color with phosphotungstate in the absence of sulfite   Cystine gives a blue color in the presence of sulfite which half reduces it to cysteine   To measure the cysteine content of a protein, therefore, phosphotungstate is added to the hydrolysate in the absence of sulfite   To measure the total cysteine plus cystine content, all the cysteine is oxidized to cystine, and the color reaction with the hydrolysate is carried out in the presence of sulfite   For the estimation of total cysteine plus cystine our method is derived from that of Folin and Marenzi (1929)   There has hitherto been no method for estimating the cysteine content of proteins

HISTORICAL

Protein SH groups have usually been detected by means of the color reaction they give with nitroprusside in presence of dilute ammonia   It was by this color test that they were first detected in coagulated egg albumin by Heffter and by Arnold   Using the same test, Arnold made a systematic investigation of the substances in tissues which contain SH groups and observed that these groups are present in two distinct forms—in the protein of tissues and in protein-free

extracts of tissues His experiments led him to believe that the substance in tissue extracts which contains SH groups is cysteine 20 years later Hopkins isolated this substance and showed it to be not cysteine but a peptide containing cysteine, which he named glutathione (Hopkins, 1921 1929) There have been many investigations of glutathione but very few of proteins as SH compounds

Heffter supposed that many denatured proteins the denatured serum proteins for example, which do not give a color reaction with nitroprusside and hence do not contain SH groups, have S-S groups which can be reduced to the SH form By treating the denatured serum proteins with the reducing agent sodium sulfite, he caused them to give a reaction with nitroprusside Protein S S groups have also been reduced by means of tin and hydrochloric acid (Arnold 1911) by soluble SH compounds (Hopkins, 1925) and by cyanide (Walker, 1925)

Protein SH groups can be oxidized into the S-S form For this purpose Heffter used sulfur which removes the hydrogen from protein SH groups and converts it into hydrogen sulfide Heffter attempted to estimate protein SH groups by measuring the amount of hydrogen sulfide formed A soluble disulfide, oxidized glutathione was later used by Hopkins instead of sulfur His experiments demonstrated clearly the existence of equilibria between glutathione in solution and sulfur groups in the protein No more than this was claimed for them (Hopkins 1925)

Direct Estimation of Protein SH Groups

This method is based on the principle first used by de Rey Pailhade (1888) and Heffter, and later by Hopkins The hydrogen of the protein sulfur groups is transferred to another sulfur compound and then estimated For this purpose we have allowed the protein to react with cystine and have then estimated the quantity of cysteine formed If this amount of cysteine is to be considered a measure of the number of SH groups present in the protein, it must be shown that *all* the protein SH groups react with cystine and *only* these That the protein SH is completely oxidized by cystine is shown by the fact that the treated protein does not give any color with nitroprusside, a sensitive reagent for SH That only SH is oxidized is indicated by the fact that only those proteins reduce cystine which give a color with nitroprusside, a fairly specific reagent for SH. Both the completeness and specificity of the oxidation are shown by the fact that the number of cystine molecules reduced by a completely reduced protein is equivalent to the number of cystine molecules found in the hydrolysate of the same protein when completely oxidized

Estimation of the cysteine formed by action of protein SH groups was first attempted by titration with iodine   This method proved inaccurate due to the low concentration of cysteine in the solutions   It was then found that cysteine in low concentrations can be estimated accurately by means of the color it develops with phosphomolybdate or phosphotungstate   Phosphotungstate has been used by Folin and Looney for the estimation of cystine [1]   In their method the color is developed in an alkaline medium and in presence of an excess of sodium sulfite which reduces the cystine and also prevents fading of the color   For our purpose sulfite could not be used to prevent fading because of its effect upon cystine   A solution of the difficulty was provided by the observation that cysteine develops a blue color with phosphomolybdate or phosphotungstate even in neutral solutions and that in these solutions fading does not occur in the absence of sulfite   Under these conditions a satisfactory proportionality over a wide range is obtained between concentration of cysteine and intensity of color   In a volume of 25 cc the quantity of cysteine can vary from 0 4 mg to 1 0 mg with a proportionate change in color intensity   A similar method for the estimation of cysteine was published by Lugg (1932) after most of the experiments described in this paper had been completed

The properties of cystine impose some limitations on the method for estimating protein SH groups by oxidation with cystine   One difficulty is that cystine is insoluble at the neutral point and becomes sufficiently soluble only when the pH is more than 9 0   Furthermore, for investigating the activity of SH groups under various conditions, a stronger oxidant than cystine is desirable   When ferricyanide, a more powerful oxidant is used, other reducing groups of proteins in addition to the SH are sometimes oxidized [2]   Indeed, one reason that the reduction of cystine by proteins is a specific test for protein SH groups is that cystine is a very mild oxidant   Strong oxidizing agents can be used in the estimation of protein SH groups if the change occurring in the protein itself is examined rather than the change in the

[1] Folin and Looney did not estimate the amount either of cystine by itself or of cysteine in the presence of cystine

[2] An investigation of some other reducing groups of proteins will be published in another paper

oxidizing agent, as is done when cystine is used   We have devised
another method based on this principle

Indirect Methods for Estimating Protein SH Groups

In these methods protein SH groups are oxidized or they are blocked
with iodoacetate, the excess of oxidizing agent or iodoacetate is re
moved, and the number of SH groups remaining in the protein is com-
pared with the number originally present

Hydrogen peroxide and potassium ferricyanide[3] can be used as
oxidizing agents   Peroxide is easier to use and is especially useful
if the cystine content of oxidized protein is to be estimated   Iodo
acetate reacts with SH groups according to the equation

$$RSH + CH_2I\ COOH \rightarrow RS - CH_2\ COOH + HI$$
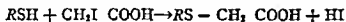
To estimate the number of protein SH groups before and after
treatment with hydrogen peroxide or ferricyanide the protein can be
exposed to cystine as described above [4]   An entirely different method,
involving hydrolysis of the protein is more convenient   The protein
is hydrolyzed, and the SH groups in the hydrolysate are estimated by
the color developed with phosphotungstate   The color reaction is
carried out as described above for the estimation of cysteine except
that a large quantity of urea is added to prevent the formation of a
precipitate   If a large number of SH groups were uncovered by
hydrolysis this method would not be accurate   Actually no such un-
covering on hydrolysis takes place   When the SH groups of a de
natured protein are oxidized or blocked, no groups at all are found
even after hydrolysis   It might be supposed that protein SH groups
would be oxidized during acid hydrolysis, and some support for this
view is provided by the fact that if cystine be added to the protein
during hydrolysis, not all of it will be recovered   And yet it can be
shown that protein SH groups are not oxidized during the acid hy
drolysis used

1 Estimation of protein SH groups by measurements of the SH
contents of the hydrolysates of reduced and untreated proteins agrees

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[3] The use of potassium ferricyanide will be described in a paper on the SH
groups of hemoglobin

[4] This procedure has not been tried after blocking SH groups with iodoacetate

with the results obtained by the cystine method which involves no hydrolysis

2 In the case of proteins of the crystalline lens which contain cysteine and no cystine the number of SH groups estimated after hydrolysis is equivalent to the quantity of cystine found in the hydrolysate of an oxidized preparation

Oxidation of SH groups while the protein is being dried preparatory to being weighed and hydrolyzed must be avoided   If neutral protein is dried at 105°C , some oxidation of its SH groups occurs   This can be prevented by keeping the protein acid while it is being dried and by shortening the time necessary for drying   For these reasons the protein is dehydrated with acid acetone before drying

Estimation of S-S Groups

In the estimation of protein S-S groups these groups are reduced to SH groups, the increase in SH groups is equal to the number of S-S groups originally present   The properties required of a reducing agent are that it should completely reduce S-S groups and that any excess of it remaining after reduction should be completely removable since the reducing agent may react with the reagent used to estimate SH

Sodium hydrosulfite and sodium sulfite do not completely reduce protein S-S groups   Indeed, when cystine is treated with sodium sulfite, as will be shown below, even in presence of a great excess of the reductant, exactly 50 per cent of the cystine is reduced   Protein S-S groups are reduced by thiol compounds, such as cysteine, reduced glutathione, and thioglycollic acid   Here an equilibrium is established

$$\text{Protein S—S} + 2\,RSH \rightleftharpoons \underset{\text{SH}}{\text{Protein SH}} + RS\text{—}SR$$
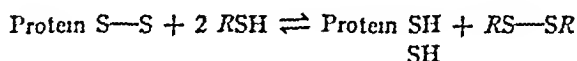
Of these thiol compounds, thioglycollic acid is the least expensive and also the most effective reducer of protein S-S groups   After a certain concentration of thioglycollic acid has been reached, doubling the concentration does not result in a further increase in number of protein SH groups   This fact indicates that the reaction has gone to completion, but does not prove that the S-S groups have been com-

pletely reduced   In the case of reduction of cystine by sulfite, it was such evidence that led Folin and Looney to believe that cystine was completely reduced when it was in fact only one half reduced, while the other half underwent some other change   To show that reduction by thioglycollic acid was actually complete, the maximum possible number of S S groups in an oxidized protein was determined by hydrolyzing it and estimating the cystine content of the hydrolysate   This number corresponds exactly to the number of SH groups present after reduction by thioglycollic acid in the denatured but unhydrolyzed protein   Since this correspondence between number of cystine molecules and number of SH groups holds for denatured proteins, in these proteins reduction of S S groups by thioglycollic acid is complete   That there is an excess of SH groups after reduction indicates that no thioglycollic acid remains adsorbed to protein, for any SH groups due to adsorbed thioglycollic acid would be included in the estimation of protein SH groups

The Cystine and Cysteine Content of Proteins

In attempting to use the Folin-Marenzi method for estimating the cystine content of proteins, we found that it can give results as much as 100 per cent too high   In this method cystine in a protein hydrolysate is reduced with sulfite, and the cysteine formed is estimated by means of the blue color which it produces with phosphotungstate   Folin and Looney believed they had shown, as has been mentioned, that the reduction of cystine by sulfite is complete and that consequently any cysteine present in the protein hydrolysate could simply be included in the "cystine" analysis   In the reaction between sulfite and cystine we find, however, that exactly 50 per cent of the cystine is reduced   If a protein hydrolysate contains cystine and cysteine, no error in analysis occurs because a cystine standard is used for color comparison   When, on the contrary, an hydrolysate contains cysteine and no cystine, an error of 100 per cent will be made, if a cystine standard is used   One way to avoid this error is to oxidize any cysteine present to cystine before treating the latter with sulfite   This can be done by denaturing the protein and oxidizing its SH groups with hydrogen peroxide before hydrolysis, for no SH groups are uncovered during hydrolysis   In this way the total cystine and

cysteine of a protein may be estimated in the form of cystine   If the denatured protein does not give a positive nitroprusside reaction, it contains no cysteine and preliminary oxidation is therefore not necessary   Most of the proteins analyzed by Folin and Marenzi contain no cysteine so that their cystine estimations of these proteins are correct   Even in the case of egg albumin which contains some cysteine their analysis is correct because drying of protein by their procedure may oxidize it   If, following the suggestion of Marenzi (1930), proteins are hydrolyzed without being dried, the estimation of cystine in egg albumin would be 50 per cent too high

The evidence that only one-half of the cystine in a solution is reduced by sulfite will now be given   Folin and Looney observed that when cystine is treated with increasing amounts of sulfite, a point is reached at which a further increase in concentration of sulfite does not cause an increase in intensity of color when the reduced cystine is mixed with phosphotungstate   Under these conditions, they concluded, cystine is completely reduced   We compared the color produced with phosphotungstate by such a cystine preparation with that produced by an equivalent amount of pure cysteine and found that in the presence of sulfite the cystine forms a color exactly twice as intense as does the equivalent amount of cysteine   This result published in a preliminary communication (Mirsky and Anson, 1930) has been confirmed by Clarke (1932), and he has also shown that the portion of cystine not reduced by sulfite is oxidized   Clarke states that he has been able to confirm the observation that a more intense color is produced by cysteine than by an equivalent amount of cystine in a general way only, for he obtained somewhat less than 100 per cent difference in color intensity   His failure to obtain a difference of as much as 100 per cent may be due to impurities in his cysteine preparations   Cysteine is prepared by the reduction of cystine and completeness of reduction must be tested by titration

The fact that only one-half the cystine in a protein hydrolysate is reduced by sulfite makes it possible to estimate the cysteine content of a protein by comparing the colors the hydrolysates of oxidized and non-oxidized protein produce when treated with sulfite and phosphotungstate   If the latter contains no cysteine the two colors should be of the same intensity, if it contains cysteine and no cystine the

color given should be twice that given by the oxidized form, and in the case of a protein containing both cystine and cysteine, the color should be between once and twice that given by the oxidized form

A better way to estimate cysteine is to dispense with sulfite and so not include cystine in the analysis This method has already been described as part of the procedure for estimating protein SH groups Estimations of cysteine by the two methods agree for some proteins (egg albumin, and reduced serum albumin, for example) but disagree for the proteins of muscle and of the crystalline lens For these proteins cysteine estimations made by reducing the hydrolysate with sulfite and then developing color with phosphotungstate in an alkaline solution are higher than those made without sulfite and at the neutral point The estimates made in alkali must be erroneous for in some instances more cysteine appears to be present than can be accounted for by the cystine found in the same proteins after oxidation Cysteine estimations made by developing a color with phosphotungstate at the neutral point appear to be reliable for in most proteins investigated the cystine content of completely reduced proteins is found to be equivalent to the cystine content of the oxidized proteins

The cystine content of a protein is obtained by subtracting its cysteine content from the total cystine plus cysteine content of oxidized protein

EXPERIMENTAL

I The Denatured Protein Preparations Used

(a) *Serum Albumin* —Denatured serum albumin is prepared in the form of a dry powder by the acid acetone procedure (Anson and Mirsky, 1931)

(b) *Egg Albumin* —Heat-coagulated egg albumin is prepared by dissolving about 1 gm of crystalline egg albumin, suspended in the ammonium sulfate solution with which it was crystallized in 100 cc water and heating with gentle stirring at 90°C until all the protein is insoluble The mixture is diluted with 125 cc of water, centrifuged, the supernatant fluid discarded, and the precipitate rubbed into a fine paste by adding a little water To the suspension are added 10 cc of a 20 per cent sodium sulfate solution and enough water to make a volume of 225 cc. After stirring the suspension mechanically for 5 minutes it is centrifuged and the supernatant fluid is discarded

(c) *Edestin* —1 gm of the dried crystalline preparation made by Hoffmann La Roche is dissolved in 200 cc. of 0 01 N HCl and then coagulated by adding

20 cc. of concentrated trichloracetic acid solution (trichloracetic acid dissolved in an equal weight of water)   The mixture is centrifuged and the supernatant fluid is discarded

(d) *Proteins of Halibut Muscle* —5 gm (wet weight) of minced frozen muscle are ground in a mortar with 50 cc of 5 per cent trichloracetic acid   The mixture is diluted to 250 cc with 5 per cent trichloracetic acid, stirred, and then centrifuged   The supernatant fluid is discarded

(e) *Proteins of the Crystalline Lens* —Six crystalline lenses, taken from the eyes of oxen, are ground in a mortar until they form a homogeneous jelly   5 per cent trichloracetic acid is added gradually and with mixing until a volume of 225 cc is reached   The suspension is centrifuged and the supernatant fluid is discarded

II *Estimation of Cysteine Content of Protein Hydrolysate*

(a) *Drying of Protein Preparations* —A preparation is first freed of any contaminating substances that may be present by washing with trichloracetic acid The protein in a 250 cc centrifuge flask is mixed with 200 cc water and to the mixture are added 20 cc of concentrated trichloracetic acid (trichloracetic acid dissolved in an equal weight of water)   After centrifuging, the supernatant is discarded, and the process is repeated as many times as may be needed   The trichloracetic acid precipitate is mixed with 200 cc acetone, stirred mechanically for 5 minutes, and then 1 cc. concentrated HCl is added to the suspension   After centrifuging, the protein precipitate is stirred with 100 cc acetone to which 5 or 6 drops of HCl are added   The acetone is decanted again, and much of what adheres to the protein is removed by suction while the flask is immersed in hot water   When the protein has reached the consistency of a thick dough, it is ground in a hot mortar until the odor of acetone has disappeared   The fine powder is now dried in an oven at 108° for 30 minutes, when a constant weight is reached

(b) *Hydrolysis*—50 to 700 mg of dried protein are transferred to a 50 cc Kjeldahl flask   5 cc of 6 N H_2SO_4 and 1 cc butyl alcohol are added   The flask is attached to a reflux condenser and placed on a sand bath where it is gently boiled for 15 hours   The condenser is then disconnected, the butyl alcohol is boiled off, the contents in the flask cooled under the tap, transferred to a 25 cc volumetric flask, and brought to this volume by dilution with water   After filtration the cysteine content of the hydrolysate may be estimated   The use of a color filter in the colorimetric comparison obviates the necessity of decolorizing the hydrolysate   If insufficient protein is available, only 2 cc 6 N H_2SO_4 need be used for hydrolysis and after hydrolysis this is diluted to 10 cc

(c) *Color Formation* —The volume of hydrolysate used, from 1 to 5 cc , should contain about 0.5 mg cysteine   If less than 5 cc are used the volume is brought to 5 cc by addition of 1 N H_2SO_4 To this are added 16 cc of concentrated urea solution (urea dissolved in an equal weight of water), 4 cc of 3.4 M K_2HPO_4 — KH_2PO_4 pH 6.8 buffer and finally , with mixing, 1 cc phosphotungstic acid   After an interval of 5 minutes the intensity of the blue color is compared with a standard

in a colorimeter   The standard used is a blue glass   the blue of which need not
closely match that of the solutions used   for a red filter is attached to the eye piece
of the colorimeter   The blue glass is calibrated by comparison with the blue
color that a standard cysteine solution produces with phosphotungstate   Phos
photungstic acid is prepared according to the directions of Folin and Marenzi

(d) *Preparation of a Cysteine Solution for Calibrating the Blue Glass Used in
Colorimetric Comparisons* —The cysteine content of the solution to be used is first
estimated   Cysteine is treated with an excess of ferricyanide and the quantity
of ferrocyanide formed is estimated colorimetrically as Prussian blue   60 mg
of cysteine bydrochloride are dissolved in 100 cc 0 01 N H_2SO_4 To 1 cc are
added 0 25 cc 1 M $K_2HPO_4 - KH_2PO_4$ solution pH 7 2 0 2 cc 0 5 M $K_3Fe(CN)_6$
and the mixture is allowed to stand for 2 minutes   Then 18 cc water, 0 5 cc. 1 N
H_2SO_4, and 5 cc of a solution containing ferric sulfate and gum ghatti   At the
same time Prussian blue is formed from a known quantity of ferrocyanide   A
solution of $K_4Fe(CN)_6$ in water is prepared containing 1 73 mg per cc, an
amount equivalent to 1 0 mg cysteine   To 0 5 cc of this solution are added
19 cc. water, 0 5 cc 1 N H_2SO_4 and 5 cc of ferric sulfate gum ghatti solution
After standing for 2 minutes the colors of the Prussian blue solutions are com
pared with the same blue glass used in measuring the blue color produced in
phosphotungstate by cysteine   To 2 cc of the cysteine solution, the concentra
tion of which is now known   are added 3 cc water, 16 cc concentrated urea solu
tion, 4 cc 3 4 M $K_2HPO_4 - KH_2PO_4$ pH 6 7 buffer and with stirring, 1 cc
phosphotungstic acid   After standing for 5 minutes the color of this solution is
compared with that of the blue glass which is to be used as a standard

The fixity of the scale on the colorimeter should be verified occasionally   For
this purpose the color of a solution of copper sulfate containing 7 gm in 100 cc.
is compared with that of the blue glass

III *Estimation of Cystine Content of Oxidized Proteins*

A denatured protein is oxidized if it gives no color reaction with nitroprusside
or if its hydrolysate does not give a blue color with phosphotungstate when
tested as described in II (c)   In the case of a protein   serum albumin for in
stance   that is oxidized in its natural state   no further oxidation is necessary

(a) *Oxidation* —To oxidize protein SH groups the denatured protein (in the
case of egg albumin about 500 mg are used) is mixed with enough 0 5 M pH 9 6
borate buffer to make the mixture definitely blue to thymol blue and enough
water is added to bring the volume to 100 cc   5 cc. of 30 per cent H_2O_2 are
added   After standing with occasional agitation for 30 minutes at room tempera
ture   100 cc water and 25 cc concentrated trichloracetic acid are added   The
suspension is centrifuged   the supernatant decanted   and enough water is added
to the precipitate so that with the aid of a rubber policeman it is rubbed first
into a thick and then a thin paste containing the albumin in a very fine state
of subdivision   Water is added to a volume of 200 cc , the mixture is stirred

mechanically from 5 to 10 minutes, and then 20 cc of concentrated trichloracetic acid are added   The washing process is repeated four times

(b) The protein is dried and hydrolyzed as described in II (a) and II (b)

(c) *Color Formation* —The volume of hydrolysate used, from 1 to 5 cc , should contain about 0 7 mg cystine   When less than 5 cc of hydrolysate is used, enough 1 N H_2SO_4 is added to bring the volume to 5 cc   To this is added 1 cc of a freshly prepared sodium sulfite solution, made by dissolving 2 gm of the anhydrous powder in 10 cc of water   After standing 1 minute, add 14 cc concentrated urea solution, 4 cc 3 4 M K_2HPO_4—KH_2PO_4 pH 6 7 buffer with stirring, 1 cc phosphotungstic acid   Color intensity is measured, after an interval of 5 minutes as in II (c)   The cystine solution for calibrating the blue glass used for color comparison is made by dissolving recrystallized cystine in 1 M H_2SO_4, 0 7 mg per cc

IV   The Cystine Content of Proteins That Are Not Completely Oxidized

This is obtained by subtracting the cysteine content of the unoxidized protein (II) from the cystine content of oxidized protein (III)

V   Protein SH Groups Estimated by the Direct Method

(a) *The Cystine Solution* —To 350 mg of recrystallized cystine are added 5 cc of water and 6 75 cc 0 5 N KOH   The cystine dissolves, and the solution is shaken in a tube which is evacuated by means of a vacuum pump   This solution must be made shortly before it is to be used, for cystine is unstable in alkaline solution

(b) *The Reaction between Protein and Cystine*—Denatured protein (about 200 mg in the case of egg albumin) is transferred to a 50 cc centrifuge tube, mixed with 45 cc of water and 2 cc of concentrated trichloracetic acid   After centrifuging and decanting the supernatant, the protein is mixed with 15 cc of a 20 per cent sodium sulfate solution and 30 cc water, stirred and centrifuged   The precipitate is mixed with 5 cc of sodium sulfate solution, and to this mixture is added the cystine solution   The tube is quickly stoppered, evacuated, filled with nitrogen (that is oxygen-free), evacuated again, again filled with nitrogen and allowed to stand at room temperature with occasional mixing for 30 minutes

(c) *Estimation of Cysteine Formed* —The protein is completely precipitated by adding 2 cc of 10 per cent sodium tungstate and then 6 cc 3 N H_2SO_4, so that the mixture is red to litmus but not blue to Congo red   After centrifuging, the total volume of the contents of the tube is measured   A measured volume of the supernatant is then removed, mixed with 4 cc of a 3 4 M KH_2PO_4 solution of pH 6 8, with 1 cc 0 5 N KOH and with enough water to bring the volume to 25 cc   To this is added 1 cc of either phosphomolybdic acid or phosphotungstic acid, and after 5 minutes the intensity of the blue color is measured in a colorimeter as described in II (c)   Phosphomolybdic acid is prepared (Wu, 1920) as follows 100 gm $Na_2MoO_4 + 2H_2O$ are dissolved in 450 cc water and to the

solution are added 15 cc. of 85 per cent H_3PO_4 and 80 cc. of concentrated HCl. The solution is boiled for 8 hours under a reflux condenser and then, without allowing the solution to cool, a few drops of bromine are added to remove the blue color The excess of bromine is driven off by boiling The solution is now brought to a volume of 1000 cc. and is ready for use

(d) *Estimation of Protein Concentration*—The protein which has been treated with cystine is washed three or four times in a 50 cc. centrifuge tube with 5 per cent trichloracetic acid separating the washings each time by centrifuging and decanting The protein is then dissolved by adding 2 to 3 cc of 0 5 N NaOH and enough concentrated urea solution (urea dissolved in an equal weight of water) to bring the volume of the solution to 25, 50, or 100 cc For a protein such as reduced serum albumin only a small quantity of protein is used, and the volume is accordingly brought to 25 cc. but when more protein is used as in the case of egg albumin, the volume is adjusted to 100 cc.

VI *Estimation of Protein SH Groups by the Indirect Method*

Either the procedure described below in (a), (b), and (c), or that in (a'), (b'), and (c') can be followed

(a) The cysteine content of a portion of the protein is estimated (II)

(a') The SH groups of one portion of protein are estimated (V)

(b) A portion of the protein is either oxidized or treated with iodoacetate Oxidation by H_2O_2 is described in III (a)

Before the protein is treated with iodoacetate, the trichloracetic acid precipitate of denatured protein (containing about 1 gm of protein) is neutralized The precipitate is stirred with a mixture containing 100 cc. 1 M K_2HPO_4–KH_2PO_4 solution of pH 7 6, 75 cc of water, and 25 cc. saturated ammonium sulfate. After centrifuging the protein suspension and decanting the supernatant the precipitate is mixed with a solution containing 60 cc 0 5 M K_2HPO_4–KH_2PO_4 solution of pH 7 3 and 33 cc. 0 1 M iodoacetate (iodoacetic acid neutralized with phenol red as indicator) The mixture remains at room temperature with occasional agitation for 3 hours To it are then added 100 cc. of water and 25 cc. of concentrated trichloracetic acid The protein is now washed four times as described in III (a)

(c) The protein prepared in (b) is dried, hydrolyzed, and its cysteine content is estimated (II)

(c') The SH groups of a protein prepared in (b) are estimated (V)

(d) The number of SH groups that reacted with H_2O_2 or with iodoacetate is calculated by subtracting the value found in (c) from that found in (a) or that in (c) from that in (a') For denatured proteins the values in (c) and (c') are zero

VII *Estimation of Protein S-S Groups*

(a) The cysteine content of one portion of the protein is estimated (II), or

(a') The SH groups of one portion of the protein are estimated (V)

(b) *Reduction of S-S Groups* —

Serum Albumin —200 mg of denatured serum albumin are dissolved in 5 cc water 10 cc of a saturated (at 30°C) Na_2SO_4 solution are added slowly and with mixing so that a finely divided precipitate is obtained 1 cc of redistilled thioglycollic acid is neutralized with a 0 4 N KOH solution, containing saturated Na_2SO_4, so that the mixture is just red to phenol red, and then 0 5 cc more alkali is added to compensate for the acidity of the albumin The solution is diluted to 60 cc with saturated Na_2SO_4, mixed with the albumin precipitate, and the mixture is allowed to stand in a 100 cc stoppered flask with occasional stirring for 45 minutes at 30°C At the end of this time the mixture is diluted with water to approximately 200 cc, and 20 cc of concentrated trichloracetic acid solution are added with stirring The washing process described in III (a) is repeated seven or eight times completely to free the albumin of thioglycollic acid

Reduction is carried out in presence of concentrated Na_2SO_4 to keep the albumin precipitated and so prevent any reversal of denaturation that might otherwise occur A temperature of 30°C is needed to obtain sufficiently concentrated Na_2SO_4 solutions Ammonium sulfate, which is sufficiently soluble at room temperature, cannot be used because it inhibits reduction

Egg Albumin —When denatured egg albumin is reduced about 500 mg are used

(c) The cysteine content of reduced protein is estimated (II), or

(c′) SH groups of reduced protein are estimated (V)

(d) The number of S-S groups is calculated by subtracting either (a) from (c), or (a′) from (c′)

RESULTS

Sufficient experimental results are now given to demonstrate the validity of the methods for a number of proteins It should not be assumed, however, that the methods are applicable to all proteins, for in at least one case difficulties are encountered Detailed studies to be published later, have been made of protein SH and S-S groups under various conditions Estimations of protein SH and S-S groups are recorded in terms of cysteine and cystine The methods yield results reproducible to within 10 per cent

Egg Albumin

1 SH groups of denatured proteins estimated by reduction of cystine by the protein −0 56 per cent

2 SH groups of *reduced* denatured protein by the reduction of cystine −1 13 per cent

3 S-S groups of denatured egg albumin, that is (2) minus (1), −0 57 per cent

4 Cysteine of untreated protein estimated by the reaction of the hydrolysate with phosphotungstate −0 616 per cent

5 Cysteine of denatured egg albumin oxidized with hydrogen peroxide, estimated after hydrolysis by reaction with phosphotungstate −none

6 SH groups of denatured protein by the indirect method, *i e* (4) minus (5), −0 616 per cent

7 SH groups of *reduced* denatured protein by reaction of the hydrolysate with phosphotungstate −1 15 per cent

8 S-S groups of denatured egg albumin by the indirect method, *i e* (7) minus (4), −0 534 per cent

9 Cystine content of denatured egg albumin, oxidized with hydrogen peroxide, by the Folin Marenzi procedure −1 24 per cent

10 Uncorrected "cystine" content by the Folin Marenzi method −1 93 per cent

Serum Albumin

1 SH groups detectable either before or after hydrolysis −none

2 SH groups of *reduced* denatured protein estimated by reduction of cystine, the direct method −4 57 per cent

3 S S groups of denatured protein calculated from (2), *i e* (2) minus (1), −4 57 per cent

4 Cysteine of *reduced* denatured protein by reaction with phosphotungstate −4 73 per cent

5 SH groups of *reduced* protein by the indirect method, *i e* (4) minus (1), −4 73 per cent

6 Cystine content by the Folin Marenzi method No correction is needed, due to (1) −4 85 per cent

Edestin

1 SH groups of protein denatured by trichloracetic acid and then reduced as estimated by the direct methods −1 18 per cent

2 Cysteine content of reduced denatured protein by reaction of hydrolysate with phosphotungstate −1 20 per cent

3 Cystine content of denatured protein oxidized with hydrogen peroxide −1 24 per cent

Mixed Proteins of the Crystalline Lens

1 SH groups of denatured proteins estimated by the direct method —1 23 per cent

2 Cysteine of the hydrolyzed proteins after the denatured, but unhydrolyzed, proteins were treated with iodoacetate —none

3 Cysteine content of hydrolyzed, untreated proteins —1 24 per cent

4 SH groups of denatured proteins by the indirect method, *i e* (3) minus (2), —1 24 per cent

5 Cystine content of denatured proteins after oxidation by hydrogen peroxide —1 105 per cent

6 Uncorrected cystine content of proteins by the Folin-Marenzi method—2 92 per cent

Mixed Proteins of Halibut Muscle

1 Cysteine content of hydrolyzed proteins —0 807 per cent

2 Cysteine content of hydrolysate after the denatured but unhydrolysed proteins were treated with iodoacetate —none

3 SH groups of denatured proteins by the indirect method, *i e* (1) minus (2), —0 807 per cent

4 Cystine content of denatured proteins oxidized by hydrogen peroxide —1 16 per cent

5 Uncorrected "cystine" content by the Folin-Marenzi method —1 94 per cent

6 Cystine content reported by Sullivan and Hess (1931) using the Sullivan method —1 13 per cent

SUMMARY

1 Methods have been described for reducing protein S-S groups, for oxidizing protein SH groups, and for estimating protein S-S and SH groups

2 It has been found necessary in estimating the cystine content of proteins by the Folin-Marenzi method to take into account any cysteine that may be present

3 A method for estimating the cysteine content of proteins has been described

4 With these methods, estimations have been made of the S-S and

SH groups and of the cystine and cysteine contents of a number of proteins

5 In a denatured, but unhydrolyzed protein, the number of S S and SH groups is equivalent to the quantity of cystine and cysteine found in the protein after hydrolysis

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PRODUCTS OF THE OXIDATION OF THIOSULFATE BY BACTERIA IN MINERAL MEDIA*

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In recent publications the author has discussed the isolation and cultivation of some bacteria which oxidize thiosulfate (27, 28) Polythionates were not detected among the products of oxidation by strictly autotrophic or facultative autotrophic bacteria but were found to be present in the products of several heterotrophic organisms including Cultures T and K of Trautwein (29) The reaction of media supporting the autotrophs became acid during growth, the heterotrophs decreased the acidity

There is very limited information concerning the products of oxidation of sulfur materials by the sulfur bacteria Waksman and Starkey determined that sulfur and thiosulfate become oxidized completely to sulfate by *Thiobacillus thiooxidans*, although some sulfur may be precipitated from thiosulfate by secondary reactions due to the high acidity which develops in the medium (36, 25) Jacobsen's results show that sulfur is oxidized to sulfate by *Thiobacillus thioparus* without the accumulation of intermediate products (12) Van Niel found that under favorable conditions the purple sulfur bacteria oxidize sulfide, sulfur, sulfite, and thiosulfate completely to sulfate although, where the process has not gone to completion, there is much sulfur in the cells and medium during the oxidation of sulfide (34) The green sulfur bacteria oxidize sulfide only to sulfur (34) In most other reports no attempt has been made to account for all of the oxidation products of the sulfur materials In the experiments reported in the following pages, detailed analyses have been conducted in an attempt to determine the exact nature of the products formed by some

bacteria oxidizing thiosulfate and the quantitative relationships between the products and the material oxidized   The characteristics of the organisms used have been discussed in preceding papers (27–29) The cultures include (1) a strict autotroph which is considered to be *Th thioparus*, (2) a facultative autotroph seemingly a hitherto undescribed culture which has been named *Thiobacillus novellus*, and (3) B, T, and K, three heterotrophic cultures reacting qualitatively alike upon thiosulfate   T and K are Trautwein's cultures described by him as facultative autotrophic bacteria (31, 32)

The media used for cultivating the bacteria and the analytical procedures involved in the following studies are recorded toward the end of this report

<div align="center">EXPERIMENTAL</div>

Products Formed by Cultures B, T, and K —Table I includes some of the results obtained with Culture T   Only averages of determinations are given and, in order to condense the data, the determinations of the controls and blanks have been subtracted where this was necessary   The pH increased with advance in development   The titrations with iodine before and after treatment with KOH indicate qualitatively that polythionates were formed   Practically all of the sulfur remained in solution during the 27 days, with not over 0 8 per cent appearing as elemental sulfur at any period of development   This accounts for only a very slight portion of the thiosulfate which was decomposed since only 4 0 per cent of the thiosulfate sulfur still remained after 27 days   Very small amounts of sulfate sulfur appeared as the culture aged, no sulfate being detected until more than one-quarter of the thiosulfate had been decomposed   It is very apparent that polythionates were the principal products of oxidation of thiosulfate   Tetrathionate was formed in greatest abundance associated with low and varying quantities of both tri- and pentathionates   All of the sulfur appears to be accounted for in the various products which were determined, within the range of the experimental error   These quantities were calculated on the basis of the total sulfur determined by the sum of the soluble and insoluble sulfur

Cultures B and K transformed thiosulfate much the same as Culture T   The reaction became alkaline and the qualitative test for poly-

thionates was strongly positive Practically no elemental sulfur appeared, small amounts of sulfate sulfur were found during the late periods of incubation Polythionates were recovered in abundance, tetrathionate being the principal product, associated with much smaller amounts of tri- and pentathionates Culture B transformed thiosulfate somewhat slower than either T or K but reacted qualita

TABLE I

Products of Decomposition of Thiosulfate by Culture T*

Product	Amounts recovered at different periods of incubation					Percentages recovered at different periods				
	5 days	8 days	13 days	19 days	27 days	5 days	8 days	13 days	19 days	27 days
	mg	mg	mg	mg	mg					
pH†	—	7 4	8 0	8 4	8 4					
0 01N I₂ for 5 cc of culture cc	13 3	11 6	5 8	1 7	0 9					
0 01N I₂ for 5 cc after KOH cc	21 2	22 1	24 5	25 1	26 9					
Soluble S	239 6	236 5	237 6	237 6	239 9	99 9	99 6	99 2	99 2	99 4
Insoluble (elemental) S	0 3	1 0	2 0	1 9	1 4	0 1	0 4	0 8	0 8	0 6
$S_2O_3'' - S$‡	164 3	143 0	70 7	19 7	9 7	68 5	60 2	29 5	8 2	4 0
$SO_4 - S$	0	0	4 6	10 3	18 9	0	0	1 9	4 3	7 8
$S_4O_6'' - S$	13 6	0	47 6	36 7	34 2	5 7	0	19 9	15 3	14 2
$S_3O_6 - S$	31 4	45 1	93 4	144 2	158 5	13 1	19 0	39 0	60 2	65 7
$S_5O_6'' - S$	16 0	21 0	33 8	25 9	19 1	6 7	8 8	14 1	10 8	7 9
Total S soluble + insoluble	239 9	237 5	239 6	239 5	241 3					
Total S insoluble + S_2O_3 + SO_4'' + S_3O_6 + S_4O_6'' + S_5O_6''	225 6	210 1	252 1	238 7	241 8	94 1	88 4	105 2	99 6	100 2

* Controls subtracted   On basis of 100 cc of solution
† Reaction of control—pH 6 6
‡ $S_2O_3'' - S$ of control 222 4 mg

tively the same as these two bacteria in all respects In 27 days, 55 8 per cent of the thiosulfate had been decomposed by Culture B It would thus seem justifiable to conclude that Cultures B, T, and K behave similarly in a qualitative way in respect to oxidation of thiosulfate although they differ in rate of development It is not un reasonable to assume that the other heterotrophic bacteria which were recorded in a preceding report as attacking thiosulfate (29), also be

have in a similar manner   The following reaction seems most likely to represent the primary transformation and this seems to be the transformation due to bacterial action

$$2 Na_2S_2O_3 + H_2O + \tfrac{1}{2}O_2 = Na_2S_4O_6 + 2 NaOH \tag{1}$$

This indicates why the pH increases as oxidation progresses, since free hydroxyl ions would be obtained   It is believed that products other than tetrathionate which were obtained, represent secondary products originating by chemical decomposition   Debus, among others, discusses in detail the transformation of polythionates in a chemical way and supplies adequate evidence to justify this conclusion (6)   Polythionates are unstable in alkaline and neutral solutions and undergo various changes   When put into solution, potassium tetrathionate breaks down to the potassium salts of penta- and trithionate

$$2 K_2S_4O_6 = K_2S_5O_6 + K_2S_3O_6$$

Potassium pentathionate breaks down to potassium tetrathionate and sulfur

$$K_2S_5O_6 = K_2S_4O_6 + S$$
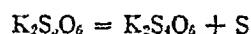
Potassium trithionate decomposes to products which finally become potassium salts of penta- and tetrathionate, potassium sulfate, and sulfur dioxide

$$5 K_2S_3O_6 = K_2S_5O_6 + K_2S_4O_6 + 3 K_2SO_4 + 3 SO_2$$

Sulfur dioxide would disappear if formed in the culture solutions by oxidation to sulfate   Thus all of the products recovered from the solutions containing Cultures B, T, and K can be accounted for by assuming that tetrathionate was initially formed by bacterial action and, by subsequent chemical reactions, gave rise to varying amounts of tri- and pentathionate, sulfate, and elemental sulfur   The relative proportions of these secondary products would vary depending upon the pH, rate of formation of tetrathionate, and interval of time during which the reactions took place   The secondary reactions are also undoubtedly responsible for the drop in pH of the culture solutions after the initial rise during prolonged incubation   This change is

particularly prominent in poorly buffered media as shown previously (28)

Trautwein stated that his cultures produced not only tetrathionate but also dithionate   The results reported above show that there is no likelihood that dithionate is produced   He failed to analyze his cultures for either of these products and speculated upon their pro

TABLE II
Products of Decomposition of Thiosulfate by Th novellus*

Product	Amounts recovered at different periods of incubation					Percentages recovered at different periods				
	5 days	8 days	13 days	19 days	27 days	5 days	8 days	13 days	19 days	27 days
	mg	mg	mg	mg	mg					
pH†	—	7 4	7 4	7 2	7 2					
0 01N I₂ for 5 cc of culture cc	17 6	17 1	16 0	15 5	14 8					
0 01N I₂ for 5 cc after KOH cc	17 6	17 2	16 1	15 6	14 8					
Soluble S	238 2	237 3	237 0	238 5	237 3	100 0	100 0	100 0	100 0	100 0
Insoluble (elemental) S	0	0	0	0	0	0	0	0	0	0
$S_2O_3'' - S‡$	217 9	211 6	198 8	191 9	182 8	91 5	89 2	83 9	80 5	77 0
$SO_4'' - S$	8 6	18 3	30 3	38 4	48 4	3 6	7 7	12 8	16 1	20 4
$S_3O_6'' - S$	—	—	0	0	0	—	—	0	0	0
$S_4O_6'' - S$	—	—	0	0	0	—	—	0	0	0
$S_5O_6'' - S$	—	—	0	0	0	—	—	0	0	0
Total S soluble + insoluble	238 2	237 3	237 0	238 5	237 3					
Total S insoluble + SO₄″ + S₂O₃″ + S₄O₆″ + S₅O₆	226 5	229 9	229 1	230 3	231 2	95 1	96 9	96 7	96 6	97 4

* Controls subtracted   On basis of 100 cc of solutions
† Reaction of control—pH 7 9
‡ $S_2O_3'' - S$ of control—223 9 mg

duction   Furthermore, he presumed that there is a constant ratio between the amounts of sulfate and polythionates produced but it now seems more likely that the ratio between any or all of the polythionates and the sulfate varies continuously depending upon the speeds of the primary and secondary reactions and has no direct relationship to the transformation effected by the bacteria

Products Formed by Th novellus

As observed from Table II the products of *Th novellus* include none of the polythionates   The qualitative test was negative at all periods and the quantitative determinations gave equally negative results when made at the last three incubation periods   The drop in pH during growth is accompanied by a continuous increase in sulfate   No elemental sulfur was formed, although in some other cases where the pH of the culture medium dropped close to 5 0, slight amounts of sulfur were found in the deposit on the bottom of the flask   This probably originates by secondary reaction through decomposition of thiosulfate in the acid medium   All of the sulfur of the decomposed thiosulfate appeared as sulfate in the experiment reported in Table II   The determinations for soluble sulfur appear to give somewhat high results as in some of the data recorded in Table I   However, the sum of the sulfur as thiosulfate and sulfate in all cases exceeded the thiosulfate sulfur of the uninoculated solutions

The results reported in Table II are verified by the results in Table V   The only product of oxidation appears to be sulfate   The culture was incubated for a longer period than that used for the determinations reported in Table II and consequently somewhat more of the thiosulfate became oxidized before the experiment was terminated   As regards oxidation of thiosulfate, this culture resembles *Th thiooxidans* which also effects complete oxidation   The two organisms differ in the range of pH over which the reaction takes place and in speed of oxidation   Furthermore, *Th thiooxidans* is a strict autotroph   The following reaction accounts for the transformation

$$Na_2S_2O_3 + H_2O + 2 O_2 = Na_2SO_4 + H_2SO_4 \qquad (2)$$
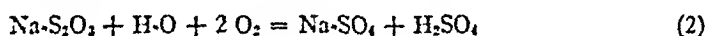
From this it is apparent why a pronounced increase in acidity accompanied oxidation and growth

Products Formed by Th thioparus

Results of determinations on solutions supporting development of *Th thioparus* are presented in Table III   In most of the solutions reported in Experiment 1 of Table III the thiosulfate had been completely decomposed and the reactions had apparently gone to completion   The results in Experiment 2 cover the periods of incomplete decomposition

As growth progressed the reaction became increasingly more acid, reaching pH 4 4 This is a lower pH than is tolerated by Cultures

TABLE III

Products of Decomposition of Thiosulfate by Th thioparus*

Age pH†	0.01N I₂ for 5 cc. of culture	0.01N I₂ for 5 cc. after KOH	Soluble S	Insoluble (elemental) S	S_2O_3—S	SO_4—S	Total S (soluble + insoluble)	Total S (insoluble + S_2O_3 + SO_4)	Products of reaction recovered as elemental S	Products of reaction recovered as SO_4—S
Experiment 1‡											
days	cc.	cc	mg	mg	mg	mg	mg	mg	per cent	per cent
4	7 2	16 8	16 9	242 5	17 4	208 7	25 6	259 9	251 7	40	60
10	6 2	0 3	0 3	146 6	92 6	2 5	144 4	239 2	239 5	39	61
12	4 4	0	0	146 0	101 2	0	144 4	247 2	245 6	41	59
14	4 4	0	0	145 7	97 0	0	143 6	242 7	240 6	40	60
14	4 4	0	0	148 3	104 6	0	145 3	252 9	249 9	42	58
17	4 4	0	0	152 3	98 4	0	147 0	250 7	245 4	40	60
18	4 4	0	0	151 7	102 6	0	143 0	254 3	245 6	42	58
20	4 4	0	0	151 1	94 2	0	145 0	245 3	239 2	39	61
20	4 2	0	0	151 4	101 2	0	145 0	252 6	246 2	41	59
21	4 4	0	0	150 9	101 7	0	147 0	252 6	248 7	41	59
21	4 4	0	0	150 9	98 6	0	147 6	249 5	246 2	40	60
22	4 4	0	0	151 4	96 7	0	147 0	248 1	243 7	40	60
24	4 4	0	0	151 7	97 1	0	148 7	248 8	245 8	40	60
Experiment 2§											
4	7 2	15 5	15 5	216 1	12 8	191 9	21 0	228 9	225 7	38	62
6	7 2	14 6	14 7	208 7	19 0	180 6	28 8	227 7	228 4	40	60
8	7 0	12 3	12 4	197 2	31 6	152 2	43 3	228 8	227 1	42	58
11	7 0	6 1	6 1	162 6	64 9	74 1	88 3	227 5	227 3	42	58
14	6 0	0	0	128 8	91 8	0	129 2	220 6	221 0	42	58
General averages										40 5	59 5

* Controls subtracted On basis of 100 cc of solution

† Reaction of control—pH 7 8

‡ S_2O_3″ — S of control 244 4 mg

§ S O₃″ — S of control 223 8 mg

B, T, K or *Th novellus* The thiosulfate is quickly oxidized without the accumulation of polythionates Nathansohn detected the for

mation of what seemed to be polythionates during his original studies
with *Th thioparus* (19)   During the course of the present studies
with *Th thioparus*, polythionates have been found only in mixed
cultures as has been discussed in a previous report (29)   It seems
most probable that the culture used in these studies is either *Th thio-
parus* or a species exhibiting identical physiological activity and vary-
ing, if at all, only in morphological characteristics

The amounts of soluble sulfur decrease rapidly as oxidation pro-
gresses due to the fact that considerable quantities of elemental sul-
fur are precipitated   The thiosulfate sulfur which is oxidized is
accounted for completely as either elemental sulfur or sulfate   The
proportions of these two products are very constant and seem to be
the same at all stages of growth   From the results reported in
Experiment 1, on the average, 40 per cent of the products of oxidation
were recovered as elemental sulfur and 60 per cent as sulfate sulfur
The averages of the data in Experiment 2 are 41 per cent elemental
sulfur and 59 per cent sulfate sulfur   Averages of all of the data show
almost exactly 40 and 60 per cent recovery as elemental and sulfate
sulfur respectively   In no case was the variation over 4 per cent
from this average   The following equation accounts for the products
in the ratios obtained

$$5 Na_2S_2O_3 + H_2O + 4 O_2 = 5 Na_2SO_4 + H_2SO_4 + 4 S \qquad (3)$$
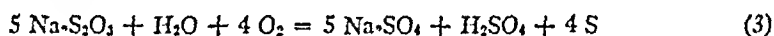
The increase in acidity is much less than by *Th novellus* per unit of
thiosulfate decomposed since four-tenths of the sulfur appears in the
elemental form   The more marked decrease in pH by *Th thioparus*
is due to the fact that the medium was poorly buffered and that
greater amounts of thiosulfate were transformed

Nathansohn believed that his cultures formed sulfate and tetra-
thionate as the initial products (19)   His results may be interpreted
otherwise (29)   Beijerinck presented the following equation to ex-
plain the transformation by *Th thioparus* and this has been copied in
most subsequent works concerned with related cultures (3, 4)

$$Na_2S_2O_3 + O = Na_2SO_4 + S \qquad (4)$$
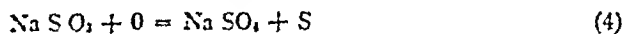
In view of the fact that the equation was apparently derived from
qualitative determinations it is interesting that it so nearly approaches

Reaction 3 which seems to explain the transformation more accurately Issatschenko and Salimowskaja applied Equation 4 to explain the reaction by their cultures (11), but this would not account for the increase in acidity which accompanied growth Salimowskaja cultured another organism which behaved much the same as *Th thioparus* but the pH increased during growth (22) Reaction 4 could not explain such a change If the reaction change resulted from the products of the thiosulfate, less complete oxidation or hydrolysis of the thiosulfate must have occurred

It might be suspected that the primary reaction by *Th thioparus* was such as 4 and that some of the elemental sulfur became subsequently oxidized to sulfate However, if such were the case, the proportion between elemental and sulfate sulfur would differ at various stages of growth of the culture No such variation has been observed Furthermore, the medium becomes acid at all stages of growth, even where decomposition was slight in the early periods This would not be expected if Reaction 4 prevailed at first

Oxidation of elemental sulfur has generally been recorded as a characteristic of *Th thioparus* (19, 3, 4, 12, 10, 11, 17) and the studies of sulfur oxidation by the strain of this organism used in the present work strengthen this conviction (28) Furthermore, the pronounced decrease in pH recorded in Experiment 1 of Table III between the 10th and 12th day might hardly be expected to have been produced as a result of oxidation of the small amount of thiosulfate remaining at the 10th day The oxidation of some elemental sulfur could account for this However, the phosphate buffers are not particularly effective between pH 6 0 and 4 0 and relatively small amounts of acid could have caused the drop in pH In case elemental sulfur was attacked, very little was oxidized since the ratio of elemental sulfur to sulfate sulfur was not altered perceptibly In case it is found that small amounts of the precipitated sulfur do become oxidized, it may be that Beijerinck's reaction will prove to be valid It would seem unwise to state that *Th thioparus* and related organisms all oxidize thiosulfate according to Reaction 3, but under the conditions of the experiments recorded in this report, this reaction has been observed with no appreciable variation irrespective of the completeness of the degree of oxidation of thiosulfate

In a recent contribution, von Deines claims that the material which is precipitated by the sulfur bacteria is not sulfur, but highly sulfured hydrogen persulfide formed from a reaction between hydrogen sulfide and sulfurous acid which he believes are produced within the bacterial cell during oxidation of thiosulfate (35)   More evidence is needed to justify the acceptance of this theory

Nathansohn believed that the precipitated sulfur arose as a result of secondary reactions   He stated that tetrathionate or similar polythionates and sulfate are produced by the initial reaction and that the reaction between the polythionates and thiosulfate gives rise to the precipitated sulfur and part of the sulfate (19)   It was also believed by the present author that the sulfur originates by some secondary reaction (25)   This does not appear to be the case since, if it were produced by secondary chemical reaction, the ratio of elemental sulfur to sulfate sulfur would not be constant at different reactions (pH) and various stages of growth   The appearance of the sulfur is also different from that observed where sulfur is slowly precipitated from thiosulfate in media becoming acid during growth of sulfur bacteria   In the latter case, it accumulates as a fine deposit on the bottom of the flask and seldom if ever as a pellicle or in such abundance

The fact that the sulfur accumulates outside of the bacterial cells need not imply that the reaction leading to its formation also took place outside of the cells   It has been shown by van Niel that whether or not the precipitated sulfur appears within the cells is determined by the size of the cells (34)   "Thus Baas-Becking found *Thiobacillus thioparus*, growing in concentrated NaCl-solutions, to occur with sulphur both inside and outside the cells in a thiosulphate medium   Due to the high salt concentration the cells were somewhat abnormally shaped, the most important difference in this connection being that they were decidedly larger" (p 57)   On the basis of numerous observations with the purple and green sulfur bacteria, van Niel further states "These observations indicate that the ability to store the sulphur globules inside the cell is dependent only upon the size and cannot be considered of any taxonomic, or even morphological importance except in connection with cell-dimensions" (p 57)

Assimilation of Carbon

One of the most critical means of distinguishing between autotrophic and heterotrophic bacteria is to demonstrate the synthesis of organic cell substance from inorganic materials by the former   The source of carbon for bacteria existing under autotrophic conditions is the bicarbonate ion (HCO_3^-)   Abundant and even somewhat limited growth as autotrophs can be demonstrated as being characterized by an increase in the organic material in the culture medium   In relatively few instances have the cultures described as autotrophic sulfur

TABLE IV

Carbon Assimilation by Culture T*

Culture	Age	pH	Thiosulfate decomposed	Carbon content	Carbon minus control
	days		*per cent*	*mg*	*mg*
Control	14	6 6	0	1 52	
Inoculated	14	7 2	41	1 53	0 01
Control	32	6 6	0	1 57	
Inoculated	32	7 8	74	1 43	−0 14
Control	35	6 6	0	1 30	
Inoculated	35	7 8	83	1 53	0 23
Control	38	6 6	0	1 40	
Inoculated	38	7 6	78	1 40	0
	45	7 6	89	1 34	−0 06
	45	7 6	84	1 38	−0 02

* On basis of 100 cc. of culture medium

bacteria been critically examined in this way   Lieske demonstrated carbon assimilation by *Thiobacillus denitrificans* during growth upon thiosulfate (18)   Jacobsen noted increases in organic carbon during development of *Th thioparus* upon elemental sulfur (12)   Carbon assimilation by *Th thiooxidans* was found to be most efficient during growth under favorable cultural conditions (36, 25, 26)   Ayyar also recorded increases in organic carbon in media supporting this organism (1)   Most recently van Niel detected pronounced assimilation of carbon by cultures of purple sulfur bacteria during growth upon sulfide

and thiosulfate and during growth of green sulfur bacteria upon sulfide (34) The following translation of words by Duggeli in 1919 seems particularly appropriate as regards the amounts of carbon assimilated by species of *Thiobacillus* Although an exact calculation of the metabolism of thionic acid bacteria is not yet available, nevertheless it can already now be mentioned, that the amount of the newly syn-

TABLE V

*Carbon Assimilation by Th novellus**

Culture	Age	pH	S_2O_3''—S†	SO_4''—S	Carbon content	Carbon minus control	$\dfrac{S_2O_3''\text{—S decomposed}}{\text{C assimilated}}$	$\dfrac{SO_4''\text{—S formed}}{\text{C assimilated}}$
	days		mg	mg	mg	mg		
Control	11	7 8	210 6	0 6	2 02			
Inoculated	11	7 0	183 8	31 8	2 11	(0 09)	(322/1)	(346/1)
Control	23	7 8	213 1	0 6	1 67			
Inoculated	23	6 8	171 6	51 5	2 88	1 21	34/1	42/1
Control	25	7 8	214 1	0 6	1 78			
Inoculated	25	6 6	165 6	53 8	2 48	0 70	67/1	76/1
Control	36	8 0	213 4	0 9	1 38			
Inoculated	36	6 6	155 0	65 8	2 78	1 40	41/1	47/1
"	43	6 4	152 5	70 1	2 63	1 25	48/1	56/1
"	43	6 4	150 6	72 4	2 61	1 23	51/1	58/1
Average						1 16	48/1	56/1

* On basis of 100 cc of culture medium Neither polythionates nor elemental sulfur recovered in any case

† Average S_2O_3'' — S of controls—212 8 mg

thesized cell substance is very small in proportion to the amount of transformed sulfur compounds (7, p 36)

Some results of determinations for carbon assimilation by Culture T are given in Table IV Even though the control media contained very small quantities of carbon (from 1 3 to 1 6 mg per 100 cc)[1] the

[1] Actually much less organic carbon was detected in the medium Blanks on 100 cc portions of distilled water yielded 1 28 mg C as an average of three determinations A fraction of a milligram of organic carbon would thus seem to be present in 100 cc of the culture medium

TABLE VI

*Carbon Assimilation by Th thioparus**

Culture	Age	Thio-sulfate decom posed	Carbon content	Carbon minus co trol	$\dfrac{S_2O_3 - S \text{ decomposed}}{C \text{ assimilated}}$	$\dfrac{S \text{ of products formed}}{C \text{ assimilated}}$
	days	per cent	mg	mg		
Control	4	0	2 66			
"	10	0	2 28			
"	12	0	2 69			
"	17	0	2 51			
"	18	0	2 85			
Average of controls			2 60			
Inoculated	4	17	2 80	(0 20)	(179/1)	(215/1)
"	10	99	5 43	(2 83)	(85/1)	(84/1)
"	12	100	4 33	1 73	141/1	142/1
"	14	100	4 63	2 03	120/1	119/1
"	14	100	4 40	1 80	136/1	139/1
"	17	100	4 48	1 88	130/1	131/1
"	20	100	4 76	2 16	113/1	111/1
"	20	100	4 38	1 78	137/1	138/1
"	21	100	4 20	1 60	153/1	155/1
"	21	100	4 44	1 84	133/1	134/1
Control	22	0	1 64			
Inoculated	22	100	3 83	2 19	112/1	111/1
"	24	100	3 88	2 24	109/1	110/1
Control	4	0	1 24			
"	6	0	1 28			
"	8	0	1 16			
"	11	0	1 26			
"	14	0	1 32			
Average of controls			1 25			
Inoculated	4	15	1 56	0 31	103/1	109/1
"	6	21	1 68	0 43	100/1	111/1
"	8	33	1 82	0 57	126/1	131/1
"	11	67	2 33	1 08	139/1	142/1
"	14	100	3 06	1 81	124/1	1?2/1
Average				1 56	125/1	127/1

* On basis of 100 cc. of culture medium

organism was able to transform relatively large quantities of thiosulfate, as much as 89 per cent was oxidized in 45 days   In no case is there any evidence of the assimilation of significant quantities of carbon   The results obtained with Culture B were quite similar and lead to the same conclusion

Th novellus behaved quite differently than the preceding organism (Table V)   In all but the youngest culture, significant amounts of organic carbon were recovered in spite of the fact that less than one-third of the thiosulfate was decomposed in any case   The ratios of sulfur transformed to carbon assimilated are found to be close to 50 1 The ratios calculated on the basis of thiosulfate-sulfur transformed are slightly different from those based on sulfate-sulfur recovered since these two sulfur values are not identical due to errors involved in the analytical procedures   The results show definitely that *Th novellus* exists as an autotroph in mineral media and transforms inorganic carbon to organic substance in proportion to the amount of thiosulfate sulfur oxidized

Somewhat more extensive results are presented in Table VI for *Th thioparus*   These data prove that the organism assimilated carbon during oxidation of thiosulfate   As shown by the determinations of the experiment reported at the lower part of the table covering various stages of growth, the amount of carbon assimilated increases with the decomposition of thiosulfate   Data concerning the sulfur transformations in the same solutions are presented in Table III   In calculating the results in the last two columns in Table VI, some of these data were utilized   The averages give practically the same figure by both methods of calculation, the ratio of sulfur oxidized to carbon assimilated was about 125 1, with the extremes of 100 1 and 155 1 excepting the first two erratic results

Energy Relations

In considerations of the energy utilization by the bacteria, values for the heats of reactions are used   Even though these may not indicate the absolute amounts of free energy available to do work (2) they sufficiently approximate such values and for comparative purposes serve the present requirement   These values have been called "machine efficiencies" by Burk since they are the resultants of numer-

ous reactions associated with reduction of carbon dioxide, growth, respiration, and other metabolic processes (5)

The reaction for *Th novellus* may be expressed as follows

$$Na_2S_2O_3 + H_2O + 2 O_2 = Na_2SO_4 + H_2SO_4 + 211\ 1\ Cal \qquad (5)$$
$$(1 \times 255\ 9)\ (1 \times 68\ 4) \qquad (1 \times 328\ 2)\ (1 \times 207\ 2)$$
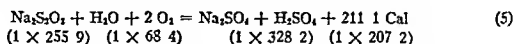
For 1 gm atom (32 gm) of sulfur in thiosulfate, 105 6 Cal are liberated   The transformation of 1 gm molecule of CO_2 with H O to glucose requires 112 2 Cal   The assimilation of 12 gm of carbon (1 gm atom) requires the oxidation of 56 times as much thiosulfate-sulfur (see Table V) or 672 gm   This amount of sulfur liberates

$$(672 \div 32)\ 105\ 6 = 2217\ 6\ Cal$$

Of this, only 112 2 Cal are accounted for in the organic carbon of the cells, therefore (112 2 − 2217 6) 100 = 5 1 per cent of the available energy is utilized

These results are quite similar to those obtained with *Th thio oxidans* during development upon thiosulfate (25)   The sulfur-carbon ratio for this bacterium averages 52 1 during development upon 1 per cent thiosulfate and 5 4 per cent of the total energy available was calculated as having been used in synthesis of cell substance

The reaction of *Th thioparus* may be stated

$$5\ Na_2S_2O_3 + H_2O + 4\ O_2 = 5\ Na_2SO_4 + H_2SO_4 + 4\ S + 500\ 3\ Cal. \qquad (6)$$
$$(5 \times 255\ 9)\ (1 \times 68\ 4) \qquad (5 \times 328\ 2)\ (1 \times 207\ 2)$$
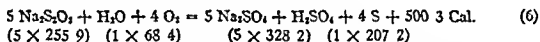
For 1 gm atom of sulfur 50 0 Cal are liberated   Using the sulfur carbon ratio of 127 1, the energy utilized for cell synthesis is calcu lated to be 4 7 per cent, a value very close to those obtained for *Th novellus* and *Th thiooxidans*

The results of Jacobsen with *Th thioparus* oxidizing sulfur to sul fate show much the same assimilation of carbon per unit of energy available (12)   Averaging his data from the fresh water and salt water strains of the bacterium, the sulfur carbon ratio is 41 1 1 Upon further calculation, using Reaction 2 of Table VII as typifying the transformation, the organic matter in cell substance accounts for 5 2 per cent of the energy which was liberated   The culture of *Th denitrificans* used by Lieske appears to be much more efficient (18)

From his data it can be calculated that 9 mg of carbon were assimilated by the oxidation of 1 0 gm $Na_2S_2O_3$ $5H_2O$, the sulfur-carbon ratio being 28 7 1   In this case 11 1 per cent of the available energy was represented in synthesized cell substance (assuming Reaction 4 of Table VII as typical)

The results of Ayyar with an organism probably identical with *Th thiooxidans* gave a somewhat higher sulfur-carbon ratio than those obtained in this laboratory   He found 40 75 1 while our results under conditions favorable for development of the bacterium were 31 8 1   He stated, "Thus the organism is much more economical in the utilization of energy "   This is apparently incorrect, since the organism oxidizing the least sulfur per unit of carbon assimilated is the most efficient   His culture shows 5 3 per cent efficiency in contrast to 6 7 per cent as determined with the cultures used in this laboratory

It is apparent from these results that in the few cases where the species of *Thiobacillus* have been carefully studied the efficiency of carbon assimilation is much the same   Quite different results are obtained with the purple and green sulfur bacteria which derive energy not only from inorganic sulfur compounds but also from light   It was found by van Niel that these bacteria assimilate far more carbon than could be assimilated by the energy supplied from the oxidation of the specific sulfur material (34)   Photosynthesis supplied a large portion of the energy utilized   Even with these bacteria, however, there is a definite proportion between the amount of the sulfur substance oxidized and the carbon assimilated

The reaction typical of Cultures B, T, and K yields very little energy

$$2 \text{ Na S}_2\text{O}_3 + \text{H}_2\text{O} + \tfrac{1}{2}\text{O} = \text{Na}_2\text{S}_4\text{O}_6 + 2 \text{ NaOH} + 21 \ 1 \text{ Cal} \qquad (7)$$
$$(2 \times 255 \ 9) \quad (1 \times 68 \ 4) \qquad (1 \times 377 \ 1) \quad (2 \times 112 \ 1)$$
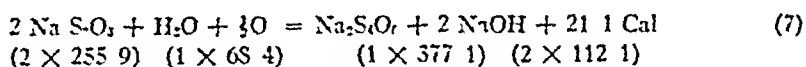
Only 5 3 Cal are liberated per gm atom of thiosulfate-sulfur   In Table VII, some of the reactions commonly applied to autotrophic bacteria have been presented together with the Calories liberated per gram atom of the significant element undergoing oxidation in the specific reactions   In all cases, the amounts of energy released are greater than that of Reaction 7 above, and are more of the order of

reactions for *Th thioparus* and *Th novellus* Only in the case of the oxidation of ferrous to ferric iron (Reaction 8, Table VII) is the energy value at a level somewhat approaching that of Reaction 7 above and there is still some question as to the actual autotrophic character of the so called iron bacteria (2, 30, 9)

Assuming it were possible for Cultures B, T, or K to develop under autotrophic conditions, very little carbon would be assimilated With the same efficiency as *Th novellus* (5 1 per cent) and with all of the thiosulfate in 100 cc decomposed (220 mg of S), only about 0 20 mg of carbon would have been transformed into organic compounds This is within the experimental error of the method used for carbon

TABLE VII

Energy Liberated by Transformations of Autotrophic Bacteria

Reaction	Significant element	Cal per gm. atom of significant element
1 $H_2S + \frac{1}{2}O_2 = S + H_2O$	S	58 5
2 $S + 1\frac{1}{2}O_2 + H_2O = H_2SO_4$	S	138 8
3 $5S + 6KNO_3 + 2H_2O = 3K_2SO_4 + 2H_2SO_4 + 3N_2$	S	122 5
4 $5Na_2S_2O_3 + 8KNO_3 + H_2O = 5Na_2SO_4 + 4K_2SO_4 + H_2SO_4 + 4N_2$	S	94 0
5 $NH_4Cl + 1\frac{1}{2}O_2 = HNO_2 + HCl + H_2O$	N	65 6
6 $HNO_2 + \frac{1}{2}O_2 = HNO_3$	N	20 9
7 $H_2 + \frac{1}{2}O_2 = H_2O$	H	34 2
8 $4FeCO_3 + O_2 + 6H_2O = 4Fe(OH)_3 + 4CO_2$	Fe	10 0

determinations, the carbon analyses by themselves are thus no absolute criterion for determining the autotrophic nature of these cultures It is believed that the combined evidence, obtained from the various studies which have been made with these cultures, is sufficient to warrant the conclusion that Cultures B, T, and K are not autotrophic, that the transformation of thiosulfate is incidental to their normal nutrition and is of no vital importance as a source of energy in their metabolism [2] It is therefore concluded that the mere disappearance or even oxidation of thiosulfate in a mineral medium through the

[2] See general conclusions concerning Cultures B T, and K as autotrophs in reference 29

agency of bacteria is insufficient evidence to justify the assumption that the organisms involved are autotrophic   The same applies to the precipitation of iron and oxidation of NH_4^+ and NO_2^- and no doubt to other transformations generally ascribed to autotrophic bacteria   It is most frequently discovered, however, that autotrophic bacteria effect more rapid and complete oxidations of the mineral substances than are common to heterotrophic organisms, providing that the environmental conditions are suited to growth of the autotrophs

Methods

The media were like those used previously (27–29) and were found suitable for studies of thiosulfate decomposition

Medium	1	2	3
Tap water	1000 gm	1000 gm	1000 gm
K_2HPO_4	2 0 "	4 0 "	6 0 "
KH_2PO_4	—	4 0 "	—
$MgSO_4\ 7H_2O$	0 1 gm	0 1 "	0 1 gm
$CaCl_2$	0 1 "	0 1 "	0 1 "
$MnSO_4\ 2H_2O$	0 02 "	0 02 "	0 02 "
$FeCl_3\ 6H_2O$	0 02 "	0 02 "	0 02 "
$(NH_4)_2SO_4$	0 1 "	0 1 "	0 1 "
$Na_2S_2O_3\ 5H_2O$	10 0 "	10 0 "	10 0 "
pH	7 8	6 6	7 8

Medium 1 was used for *Th thioparus*, Medium 2 for Cultures B, T, and K, and Medium 3 for *Th novellus*   The media were generally distributed in 250 or 300 cc amounts in Erlenmeyer flasks of 1 liter capacity   The ammonium sulfate and sodium thiosulfate were sterilized separately   In some cases 100 cc portions of the media were used in 250 cc Erlenmeyer flasks   Since about 200 cc of solution were used in the analyses, the culture solution of two or more flasks was pooled at each period of study when the small culture flasks were used   In the studies of *Th thioparus*, distilled water was used in place of tap water

The analyses were conducted as follows

I   The culture solution was made to definite volume in volumetric flasks

II   Where determinations were made for carbon content of the liquids, the method used was much the same as that which proved satisfactory for determining carbon assimilation by *Th thiooxidans* (36)   The total solution was agitated to properly disperse all suspended materials   Duplicate 100 cc portions of the unfiltered liquid were pipetted into combustion flasks, 5 cc portions of 1–4 H_2SO_4

were added and the solutions aerated for at least 1 hour in the cold to release any inorganic carbon from the solution In cases where there was thiosulfate in the culture solution, sulfurous acid was formed by the decomposition of thiosulfate in the acid solution This was trapped after leaving the reflux condenser by passing the gas stream through a solution containing 1 gm of $KMnO_4$ in 50 cc. of water This solution was renewed for each determination After aeration in the cold the units for absorbing carbon dioxide were placed in the gas chain, 5 0 gm of $KMnO_4$ were added to the combustion flasks and 50 cc of concentrated $H SO_4$ were run in slowly during which time the system was being aerated The solutions were then heated to boiling for 3 hours and the carbon dioxide from the gas stream was trapped in standard 0 05N $Ba(OH)_2$ in Truog towers in place of the tubes used in the earlier studies After completion of the combustion the excess $Ba(OH)_2$ was titrated with 0 05 N oxalic acid The media used for studies including the carbon determinations were slightly different from those listed above, $CaSO_4$ 2H O replaced the $CaCl$ and $Fe_2(SO_4)_3$ $9H_2O$ replaced the $FeCl_3$ $6H_2O$ This avoided the interference of chlorides in the determinations

III After removing the aliquots for carbon determinations, the remaining liquid was filtered through asbestos in Gooch crucibles to separate the precipitated sulfur if any from the rest of the solution Some precipitated phosphates and growth were also removed by the filtration In cases where carbon determinations were not made the entire culture solution was filtered before any analyses were performed. The filtrates were made to volume Since insignificant amounts of sulfur other than that in the elemental form were removed by filtration this sulfur is referred to as elemental sulfur in the recorded results

IV After being washed with a small quantity of distilled water, the contents of the Gooch crucible were washed into a 250 cc. beaker and total sulfur was determined as sulfate after oxidation by a bromine-carbon tetrachloride solution as recommended by Scott (24) This method proved to be very accurate and effective for oxidation of the various sulfur materials concerned in these studies Superoxol was effective as an oxidizing agent of sulfur only in strongly alkaline solutions The bromine potassium bromide oxidizing reagent suggested by Scott proved ineffective in oxidation of the sulfur compounds The total sulfur contained in the filtrates of the culture solutions was also oxidized in a similar manner 10 cc. aliquots of the filtrates being used in duplicate

V After oxidation by Br CCl_4 the total sulfur was determined as sulfate by the use of benzidine hydrochloride recommended by Treadwell and Hall (33) The method was modified in that the benzidine reagent was not strongly diluted before addition to the solution being tested which was generally made up to about 100 cc. By use of a Buchner funnel as suggested the blank was too variable since very little wash water is used It was found much more satisfactory to use Gooch crucibles with asbestos pads which could be very readily washed free of excess acid by three portions of 5 cc. of distilled water In order that the precipitated benzidine sulfate should not cake during filtration, a small amount of suspended

asbestos was added to the liquid before filtration This greatly accelerated subsequent titration with the NaOH The addition of asbestos was unnecessary with the determinations on the elemental sulfur since asbestos had been introduced in the form of the pad used for the initial filtration

VI Sulfur occurring as sulfate in the culture filtrate was also determined by the use of benzidine hydrochloride Duplicate aliquots of 10 cc were used in 250 cc beakers Since thiosulfate decomposes in acid solutions and also precipitates to a considerable extent with benzidine, this was oxidized by iodine (0 1 N) which was slowly added until a light yellow color persisted showing a slight excess of iodine The polythionates do not react with benzidine nor are they oxidized by iodine Any sulfite or sulfide which might occur in the solution would also have been oxidized by the iodine but neither of these was found in any of the culture solutions After treatment with iodine, the solutions were used for sulfate determination as under V

VII In order to determine whether or not sulfite or sulfide occurred in the solutions, the method of Kurtenacker and Bittner for the determination of thiosulfate, sulfite, and sulfide in mixtures was used (13) It is unnecessary to refer further to formation of sulfide or sulfite since neither was detected in any of the culture solutions Consequently, the iodine titration of the culture filtrate was used for the determination of thiosulfate Duplicate 10 cc aliquots were used The titration was conducted with 0 01N iodine solution using starch as the indicator in the solution acidified with acetic acid

VIII The qualitative test for polythionates depended upon their reaction with alkalis as described in a preceding paper (29) The iodine titration of the unaltered culture filtrate indicates the thiosulfate Since treatment with alkalis caused polythionates to decompose to sulfite and thiosulfate, and since these two products react with iodine, a second titration with iodine following alkali treatment will be greater than the first in case polythionates are present

Tri-, tetra-, and pentathionates were determined essentially the same as outlined by Reisenfeld and Sydow (21) involving the reactions of tetra- and pentathionates with sulfite as shown by Raschig (20), the reactions of these two polythionates with cyanide as shown by Kurtenacker and Fritsch (15, 16, see also Kurtenacker and Bittner (14)), the reaction of all three polythionates with mercuric chloride as developed by Feld and Sander (8, 23) These methods proved to be quite satisfactory but were not as desirable as methods for determining each of the polythionates separately by specific reactions had such been available

IX Tetra- and pentathionates react with sulfite as follows

$$S_4O_6'' + SO_3'' = S_3O_6'' + S_2O_3'' \tag{8}$$
$$S_5O_6'' + 2 SO_3'' = S_3O_6'' + 2 S_2O_3'' \tag{9}$$
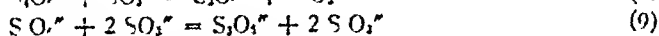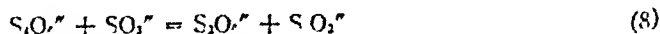
The thiosulfate produced by the reactions can be determined by titration with iodine The procedure was conducted as follows Two 10 cc aliquots of the culture filtrate were placed in 250 cc beakers and 2 cc of saturated Na_2SO_3 were

added, the solution neutralized with 0 1N KOH using pheoolphthalein as iodicator, and after standing for 5 minutes 5 cc of formaldehyde (35 per cent) were added to block the excess sulfite   The deep red colored solution was decolorized with 10 per cent acetic acid and an excess of 20 cc was added   This solution was titrated with 0 01 N iodine solution using starch as the iodicator   The iodioe titration (which may be called a) measures the original thiosulfate of the culture solutions and thiosulfate produced from reaction of tetra and pentathionates with sulfite

X   The reactions of the polythionates with cyanide are the following

$$S_2O_6'' + 3\ CN + H_2O = SO_4 + CNS + 2\ HCN + S_2O_3'' \tag{10}$$
$$S_4O_6'' + 4\ CN + H_2O = SO_4'' + 2\ CNS + 2\ HCN + S_2O_3'' \tag{11}$$
$$S_6O_6'' + 3\ CN + H_2O = SO_4'' + CNS + 2\ HCN + SO_3'' \tag{12}$$
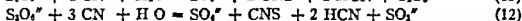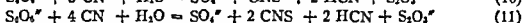
The sulfite which is formed from trithionate is blocked by formaldehyde permitting the titration of the thiosulfate from the tetra and pentathionates with iodine   In the determination two 10 cc aliquots of the culture solution are introduced into 400 cc beakers diluted to about 200 cc, and theo receive 7 cc of a 10 per cent solution of KCN   After standing for 5 minutes, 5 cc of formaldehyde (35 per cent) are added together with 5 cc of 1 to 4 H_2SO_4 The titration of this solution with 0 01N iodine solution gives a measurement of the thiosulfate of the culture solution together with the thiosulfate formed by the reaction of the tetra and pentathionates with cyanide   This value is b

XI   The polythionates and thiosulfate react with mercuric chloride as follows

$$2\ S_2O_3 + 3\ HgCl_2 + 2\ H_2O = Hg_3S_2Cl_2 + 4\ Cl + 2\ SO_4'' + 4\ H \tag{13}$$
$$2\ S_3O_6'' + 3\ HgCl_2 + 4\ H_2O = Hg_3S_2Cl_2 + 4\ Cl + 4\ SO_4 + 8\ H \tag{14}$$
$$2\ S_4O_6'' + 3\ HgCl_2 + 4\ H_2O = Hg_3S_2Cl_2 + 4\ Cl + 2\ S + 4SO_4 + 8\ H \tag{15}$$
$$2\ S_5O_6 + 3\ HgCl_2 + 4\ H_2O = Hg_3S_2Cl_2 + 4\ Cl + 4\ S + 4SO_4 + 8\ H \tag{16}$$
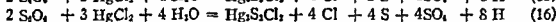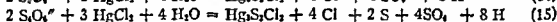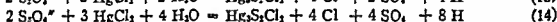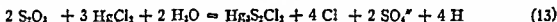
The acidity produced by these reactions is determined as follows   Two 10 cc. aliquots of the culture filtrate are placed in 400 cc beakers and the thiosulfate remaining in the solution is oxidized to tetrathionate by iodine (0 1 N), added a drop at a time until a very weak yellow color persists   The solution is made neutral to methyl orange and 100 cc. of saturated $HgCl_2$ are added and the solution heated to boiling for 20 minutes   After adding 100 cc of 4 N NH_4Cl, the solution is titrated to the original neutral color of methyl orange using 0 0714 N NaOH   This titration measures the acidity produced from tri tetra and pentathionates and the thiosulfate contained in the solution   This value is called c

Calculations were made as follows   The iodine value (mg) of the thiosulfate in the culture filtrate as determined under VII was calculated on the basis of 100 cc. of solution (m)   This was subtracted from the iodine value (mg) calculated from (a) of the determination by the sulfite method under IX and based on 100 cc of solution   This difference divided by the atomic weight of iodine is the mg atoms of iodine required for reaction with the thiosulfate formed from tetra and pentathionates by reaction with sulfite (called x)

In a similar manner the iodine value m is subtracted from the iodine value (mg) calculated from b in the determination by the cyanide method under X and based on 100 cc of solution   This difference divided by the atomic weight of iodine is the mg atoms of iodine required for reaction with the thiosulfate formed from tetra- and pentathionates by reaction with cyanide (called y)   Then $x\text{-}y = \alpha$, the mg atoms of iodine from the reaction of the excess thiosulfate formed from pentathionate by reaction with sulfite as in Reaction 9   This excess of thiosulfate is shown by inspection of the reactions under IX and X to be 1 molecule of thiosulfate for each molecule of pentathionate   The following reaction shows that in titration of thiosulfate with iodine, 1 atom of iodine reacts with 1 molecule of thiosulfate

$$2 S_2O_3'' + I_2 = 2 I' + S_4O_6'' \tag{17}$$

Consequently the value of α equals the millimols of pentathionate found in 100 cc of the culture filtrate   The sulfur content of this number of millimols of pentathionate is found by multiplying by 160 3, the weight of the 5 atoms of sulfur in the molecule

In the determination of tetrathionate $y\text{-}\alpha = \beta$, the mg atoms of iodine required for reaction with the thiosulfate formed from tetrathionate by cyanide   Since 1 atom of iodine reacts with 1 molecule of thiosulfate which in turn is formed from 1 molecule of tetrathionate (Reaction 10), the factor β also equals the millimols of tetrathionate   Then $\beta \times 128\ 24$ is the mg of sulfur as tetrathionate in 100 cc of culture solution

Calculations of trithionate involve the use of the determinations from reactions under XI   The mg of hydrogen equivalent to the thiosulfate previously determined in 100 cc of culture filtrate (calculated from Reaction 13) is subtracted from the mg of hydrogen in 100 cc of culture filtrate reacting with alkali in the mercuric chloride method (XI) and calculated from c The difference is the hydrogen (mg in 100 cc of solution) from reactions of tri- , tetra- , and pentathionates according to Reactions 14, 15, and 16   This value (called z) is also the mg atoms of hydrogen   The following equation permits the calculation of the millimols of trithionate (γ)

$$\gamma \text{ or the millimols of trithionate} = \frac{z - 4\alpha - 4\beta}{4}$$

Then $\gamma \times 96\ 18$ gives the mg of sulfur as trithionate in 100 cc of the culture filtrate

SUMMARY

Various cultures (previously described), which oxidize thiosulfate in mineral media have been studied in an attempt to determine the products of oxidation   The transformation of sodium thiosulfate by

Cultures B, T, and K yields sodium tetrathionate and sodium hydroxide, secondary chemical reactions result in the accumulation of some tri and pentathionates, sulfate, and elemental sulfur As a result of the initial reaction, the pH increases, the secondary reactions cause a drop in pH after this initial rise The primary reaction yields much less energy than the reactions effected by autotrophic bacteria No significant amounts of assimilated organic carbon were detected in media supporting representatives of these cultures It is concluded that they are heterotrophic bacteria

Th novellus oxidizes sodium thiosulfate to sodium sulfate and sulfuric acid the pH drops progressively with growth and oxidation Carbon assimilation typical of autotrophic bacteria was detected, the ratio of sulfate sulfur formed to carbon assimilated was 56 1 It is calculated that 5 1 per cent of the energy yielded by the oxidation of thiosulfate is accounted for in the organic cell substance synthesized from inorganic materials This organism is a facultative autotroph

The products of oxidation of sodium thiosulfate by *Th thioparus* are sodium sulfate, sulfuric acid, and elemental sulfur, the ratio of sulfate sulfur to elemental sulfur is 3 to 2 The pH decreases during growth and oxidation The elemental sulfur is produced by the primary reaction and is not a product of secondary chemical changes The bacterium synthesizes organic compounds from mineral substances during growth The ratio of thiosulfate sulfur oxidized to carbon assimilated was 125 1, with 4 7 per cent of the energy of oxidation recovered as organic cell substance This bacterium is a strict autotroph

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RESULTS OF IRRADIATING SACCHAROMYCES WITH MONOCHROMATIC ULTRA VIOLET LIGHT

IV Relation of Energy to Observed Inhibitory Effects

By R. H. Oster and W. A. Arnold

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Quantitative treatment of the changes resulting in microorganisms after irradiation with monochromatic radiation of known frequency and intensity has yielded useful information (*cf* Gates, 1929–30, Wyckoff, 1931–32), and has recently provided the basis for some interesting theories as to the mode of action and place of absorption of radiant energy by the living cell (*cf* Crowther, 1924, 1926, Swann and del Rosario, 1932, Curie, 1929, Glocker, 1931, Lacassagne, 1930, and Holweck, 1932) However, these theories cannot be tested properly until a large amount of quantitative data is obtained under carefully controlled conditions

In the present paper certain data obtained on *Saccharomyces cerevisiae* exposed to monochromatic ultra violet radiation of different wave lengths and measured intensities (Oster, 1934–35) have been examined according to the method proposed by Mme Curie (1929), with respect to the amount of reproduction after irradiation and subsequent incubation for 36 hours

Yeast cells from 24 hour cultures were inoculated on malt agar plates, exposed in a large quartz monochromator, incubated for 36 hours at 25°C, and counted under the microscope as previously described (Oster, 1934–35) Although in most of the experiments cell counts were made solely on the basis of survival (*i e*, all cells forming two or more daughter cells were considered to be "alive"), in other experiments detailed counts permitted the cell groups to be placed in five classes (*a*) single cells, (*b*) 2-cell groups, (*c*) 3 to 4-cell groups, (*d*) 5 to 16 cell groups, and (*e*) 16 or more-cell colonies

The survival curve can then be plotted using the percentage of cell

groups in a given class as the criterion of "survival," as a function of the incident energy per unit area of receiving surface (Fig 1)

The analysis of such survival curves has been treated by Curie as follows

Let P = probability of survival, N/N_o
$p = S - 1$, where S = minimum number of quanta to kill
x = amount of energy supplied
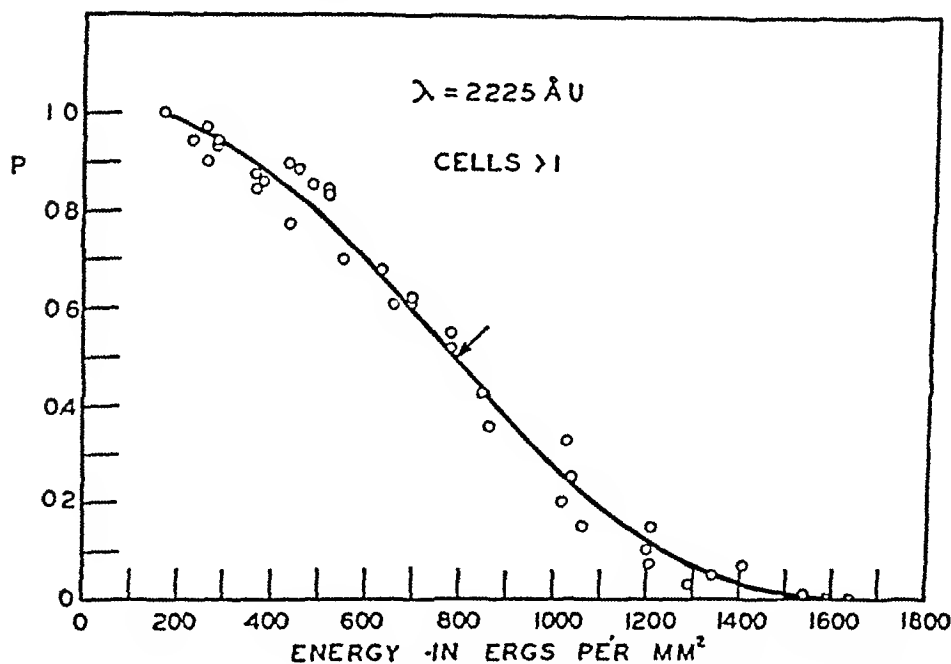
Fig 1 Survival curve of yeast cells exposed to monochromatic ultra-violet radiation of wave-length 2225 Å u   The survival ratio N/N_o is plotted as a function of the incident energy   The criterion used in this curve is the ability of the irradiated cell to form one or more daughter cells during 36 hours of subsequent incubation at 25°C   The inflection point of the curve is indicated by an arrow Each point plotted represents the counting of from 150 to 300 cell groups per irradiated area

Then if we plot P as a function of x, Curie shows that at the point of inflection we have

$$- x\frac{dP}{dx} = \div \zeta(p) = \div \frac{p^p e^{-p}}{(p-1)!},$$

and by Stirling's expression for the factorial we reach finally

$$-x\frac{dP}{dx} = \sqrt{\frac{p}{2\pi}} = \sqrt{\frac{S-1}{2\pi}}$$

TABLE I

Data used in determining the number of quantum hits (S) involved in the inhibition of cell division in yeast exposed to ultra violet radiation of different wave-lengths according to the probability method of Mme Curie (1929) In the table x is the abscissa value of the inflection point of the survival curve $p = \phi 2\pi$ and $S = p + 1$ is threshold number of quantum hits

Wave-length	No. of new cells formed in 36 hrs.		x	Slope dp/dx	p	S
λu						
2225	More than	1	790	0 011	4 7	5 7
	"	2	700	0 012	4 1	5 1
	"	4	620	0 012	3 5	4 5
	"	15	540	0 013	3 1	4 1
2537	"	1	720	0 010	3 4	4 4
	"	2	440	0 016	2 9	3 9
		4	435	0 015	2 6	3 6
		15	360	0 017	2 3	3 3
2804	"	1	785	0 012	5 1	6 1
	"	2	490	0 015	3 4	4 4
	"	4	485	0 014	2 9	3 9
		15	410	0 0t5	2 3	3 3
2652	"	2	470	0 018	4 3	5 3
		7	3t0	0 024	3 4	4 4
2482		2	680	0 012	4 4	5 4
2378		2	1t20	0 007	4 0	5 0

Whence we can determine S, the number of quantum hits necessary to kill

On this basis the available data for the separate classes of yeast cells were plotted and a smooth curve drawn as shown in Fig 1, where the curve of survival ratio for cells irradiated at the wave length 2225 Å u , using the criterion of ability to form one or more daughter cells in 36 hours, typifies the shape of the curves obtained By determin-

ing the inflection point the values of ι and the slope of the curve were obtained and S calculated from the expressions given above (Table I)

If we neglect for the present the possible influence of other factors such as the age and the reproductive state of the cell at the time of irradiation, the values of S in Table I tend to indicate that the process of inhibiting and "killing" of yeast cells by ultra-violet energy follows a multiple quantum hit to kill relation, as suggested by Wyckoff and Luyet (1931), and that the minimum number of quantum hits varies from approximately four (required before cell division is inhibited to less than fifteen daughter cells) to approximately six (required before no daughter cells are formed)   If such a relation could be definitely demonstrated it might indicate the existence within the yeast cell of several small entities each of which are involved in a future budding

It should be pointed out that too much emphasis must not be placed on the significance of the values obtained for S Each point on the plotted survival ratios involved the counting of from 150 to 300 cell groups under the high power of the microscope, but to determine the slope and inflection point of the curve for a rigorous evaluation of $\phi(p)$ hundreds of points and the counting of many thousands of cell groups would be necessary

However, an inspection of the values of S obtained seems to indicate that different numbers of quantum hits are involved in the production of different degrees of inhibition of cell division, and furthermore that the number of quantum hits increases with increase in the degree of inhibition secured

SUMMARY

Data obtained on yeast irradiated with monochromatic ultra-violet radiation has been analyzed for the number of quantum hits involved in the production of different degrees of inhibition of cell division, according to the method proposed by Mme Curie (1929)   Sufficient data are not available for a rigorous determination, but the calculated results tend to indicate that a multiple hit to kill relation is followed, that different numbers of hits are involved in the production of different degrees of inhibition, and that this number increases with increase in the degree of damage sustained

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STIMULATION BY COLD IN NITELLA

By SAMUEL E. HILL

Umrath[1] has noted that action currents are produced in *Nitella* by local application of cold [2]

In order to see just what happens the following procedure was adopted [3] Cells which had been kept for 7 to 12 days in Solution A[4] were tested, using the arrangement shown in Fig 1 All responded normally to electrical stimulation (about 120 mv D C applied by means of saturated calomel electrodes)

The cell whose record appears in Fig 2 was placed on a paraffin block at room temperature (22°C) with Solution A at *C* and *D* (the cell wall was imbibed with Solution A) Fresh Solution A at 22°C was then applied at *C*, causing no change [5] Solution A at 1°C was then applied at *C* which became more negative[6] to the extent of 13 mv , without setting up a characteristic action current this happened in about 6 per cent of the cells (the alteration ranged from 10 to 20 mv)

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[1] Umrath, K , *Protoplasma*, 1930 9, 576

[2] This had also been observed in this laboratory by W J V Osterhout and E S Harris and photographically recorded in 1927 (personal communication)

[3] The technique unless otherwise stated, is as in previous papers (Osterhout, W J V and Hill, S E , *J Gen Physiol* , 1930–31, 14, 473 1933–34 17, 87) The experiments were carried out at room temperature 22 ± 1°C The material was *Nitella flexilis* Ag

[4] This is a nutrient solution for its composition see Osterhout, W J V , and Hill S E *J Gen Physiol* 1933–34, 17, 87

[5] The solutions were poured rapidly and continuously on a strip of filter paper which passed under the cell at *C*, precautions being taken to avoid mechanical or electrostatic shock In some cases there are irregular small changes (2 to 3 mv) as the result of this procedure

[6] That this change is not due to streaming potential is shown by the fact that it does not occur if the solution is at room temperature

As the normal potential across the protoplasmic surface is outwardly directed (positive) the fact that C becomes more negative means that there is a partial loss of P D

As explained elsewhere the measured P D depends on a number of E M F 's[7] and of resistances [8] The temperature coefficients need not
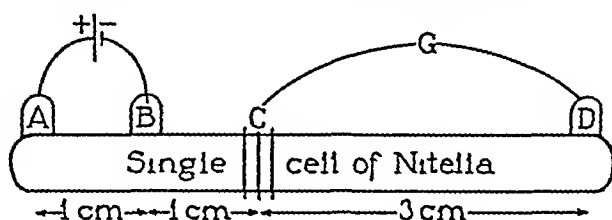
Fig 1 Arrangement for testing cells (placed on a block of paraffin) with solutions at various temperatures The electrical stimulation was applied through saturated calomel electrodes at A and B C was a flowing contact for changing solutions during recording The recording instrument (G) was a Cambridge string galvanometer with thermionic amplifier (making the measurement essentially electrostatic)

be the same for all of these Hence (even in the absence of secondary effects and of stimulation) we do not know just how much lowering of P D to expect with a given fall of temperature,[9] but it seems safe to

[7] The lowering of temperature would, of course, lower the E M F and raise the resistance Lowering the temperature from 22°C to about 3°C increased the average resistance of 10 *Nitella* cells 44 2 per cent This is the combined ohmic and polarization resistances at one spot, 1 cm long, in contact with Solution A The other contact was chloroformed The measurement of resistance was made according to the method of Blinks (Blinks, L R , *J Gen Physiol* , 1929–30, **13**, 495)

According to Bernstein (Bernstein, J , Elektrobiologie, Braunschweig, F Vieweg und Sohn, 1912, 91) the P D in muscle is directly proportional to the absolute temperature Bernstein (Bernstein, J , *Arch ges Physiol* , 1910, **131**, 589) also states that the demarcation potential of muscle changes when the temperature is altered at the intact surface, but not when the change is made at the cut end Verzár (Verzár, F , *Arch ges Physiol* , 1912, **143**, 252) repeating this experiment, found an effect also at the cut end

[8] For a discussion of E M F 's and resistances in *Nitella* see Osterhout, W J V , and Harris F S *J Gen Physiol* , 1927–28, **11**, 673

[9] Raising the temperature above that of the room does not produce much change in P D The reason for this is not clear but it may depend on the fact that as the E M F increases the resistance falls

say that it would be of the order of magnitude observed in these ex periments

When the flow of cold solution at C ceased the original P D was slowly regained [7] The whole procedure shown in Fig 2 is fairly typical for those cells which are not stimulated

As a rule the sudden application of cold Solution A stimulates the cell and a negative variation results The shape of the action curve
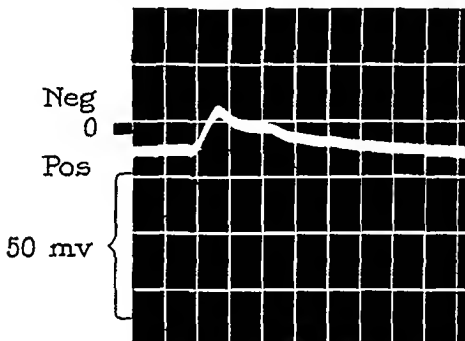
Fig 2 Photographic record of partial loss of P D in *Nitella* caused by application of Solution A at 1°C at C by flowing contact (arranged as in Fig 1) No characteristic action current was produced The cell had been kept in Solution A for 6 days The vertical lines represent 5 second intervals Room temperature 22 C

at the cooled spot differs from that at a normal spot in one or more of the following particulars (1) The ratio of the height of the first peak to the second is reduced (2) The total change of P D is greater or less at the cooled spot (3) The recovery of potential is slower at the cooled spot [10]

Fig 3 (the experiment being arranged as in Fig 4) shows the reduction of the first peak and decrease in total change of potential In

[10] Gasser (Gasser H S *Am J Physiol* 1931 97, 254) states that cooling frog nerve causes a prolongation of the spike accompanied by a considerable falling off in height and this effect increases as the temperature is lowered

this case C, D and E were in contact with 0 001 м KCl (at 23°C) The negative variation at D (middle curve) which was started by the application (at D) of 0 001 м KCl at 1°C spread along the cell in
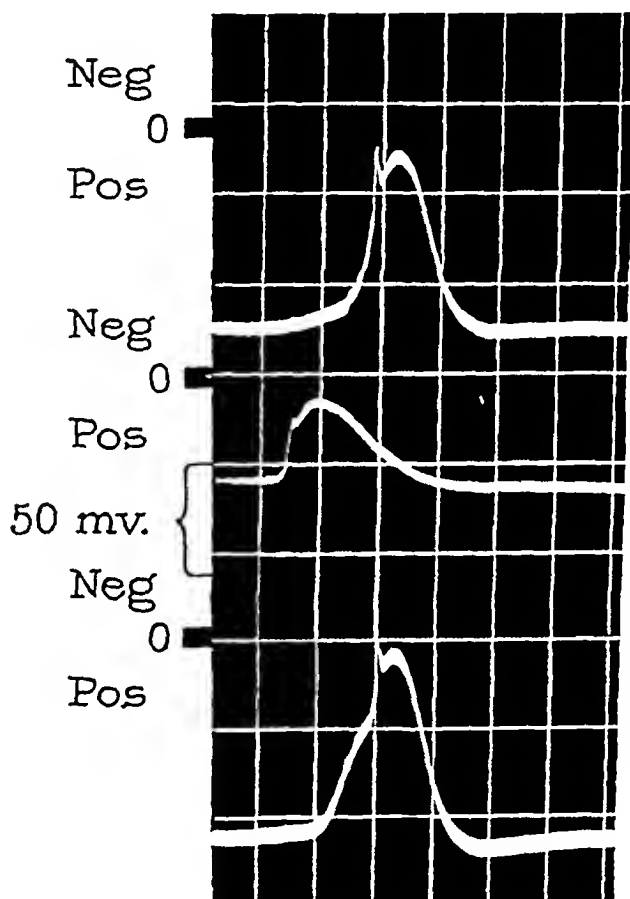
first peak is lacking at the cooled spot (middle curve) In both these records F had been killed by chloroform at the start to give monophasic responses

In Fig 3 the action current carried the P D at the normal contacts to zero or nearly so, while in Fig 5 the action current reduced the P D by only about two thirds of its value This is not unusual in *Nitella*, an action current frequently failing to discharge all the polarization of the membrane

Fig 6 (with the same arrangement, but with Solution A on contacts C, D, and E, and with 2 cm spacing between contacts) shows a
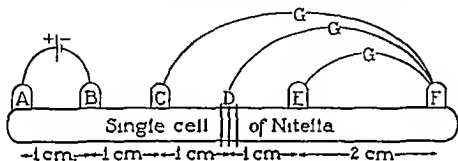
FIG 4 Arrangement for testing cells (placed on a block of paraffin) with solutions at various temperatures D is a flowing contact for changing solutions during recording F may or may not be killed All contacts are made through saturated calomel electrodes GGG represent Cambridge string galvanometers with thermionic amplifiers (the measurement is essentially electrostatic) The recording instrument was a Cambridge Type A string galvanometer in which the single string had been replaced by three tungsten wires each 5μ in diameter Careful calibration shows the deflections to be proportional to the applied voltage within the limits of the recording paper here employed

relative increase in the second action curve at the cooled spot In the first action current at D (middle curve), before D was cooled the change of P D was approximately the same as at C and E but after it was cooled (second action current) the second peak of the curve was higher by about 40 mv This in itself is not unusual, as such variations have often been observed[11] at ordinary temperatures In this case (and many other cases like it) the only difference in conditions at the three recorded contacts was the cooling at contact D In Fig 7 the variation caused by the application at D (upper curve) of

[11] The fact that the action curve goes above zero may be due to some positive P D at the common contact E

Solution A at 1° was practically the same as at contact E (lower curve) at 22°C   The reason for the occasional increase in value of the second peak will be discussed in a later communication
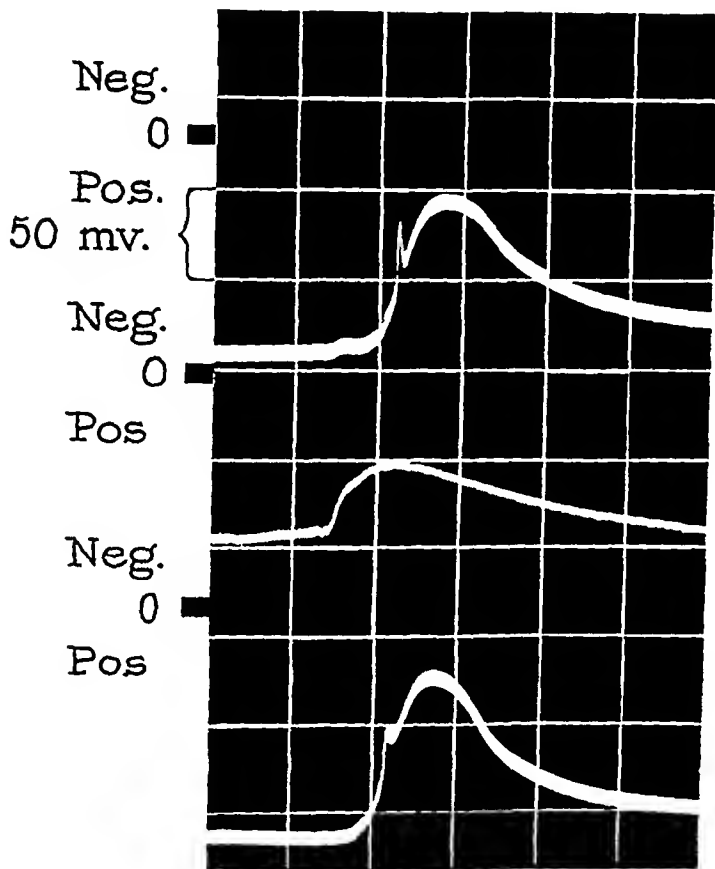
Fig 5 Photographic record of negative variations caused by the application of 0 001 M KCl at 1°C at D (Fig 4)   The top curve shows changes at C, the middle curve at D, and the bottom curve at E All contacts were 0 001 M KCl F was killed with chloroform before the experiment started   The cell was kept for 2 days in Solution A   The vertical lines represent 5 second intervals   Room temperature, 21°C

In *Chara*[12] we observe at the cooled spot a curve which shows less total change in P D , or slower recovery, or both, than at a spot at room temperature   This is shown in Figs 8, 9, and 10 [13]   Here

[12] This is *Chara coronata*, Ziz
[13] The arrangement, solutions, and distances were as in Fig 4
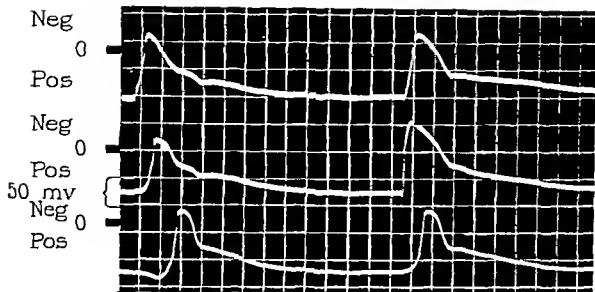
FIG 6 Photographic record of negative variations in *Nitella* The first varia
tion was caused by electrical stimulation with 120 mv D C at B (Fig 4) The
variation passed along the cell and was recorded at C (top curve) D (middle curve)
and E (bottom curve) The application at D of Solution A at 22 C (shown by
the slight disturbance on the curves 45 seconds after the record started) was with
out effect The second variation was caused by the application at D (67 seconds
after the record started) of Solution A at 1°C It passed along the cell in both
directions and was recorded at C (top curve) and E (bottom curve) F was
brought nearly to zero by application of 0 01 M KCl at F before the record started
All contacts were Solution A in which the cell had been kept for 17 days The
vertical lines represent 5 second intervals Room temperature 21°C
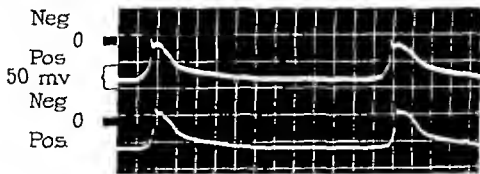
FIG 7 Photographic record of negative variations in *Nitella* The first reac
tion was caused by electrical stimulation at B (Fig 4 with C omitted) The varia
tion passed along the cell and was recorded at D (upper curve) and E (lower curve)
The second variation recorded in the upper curve was caused by the application
at D of Solution A at 1°C The negative variation passed along the cell and was
recorded at E (lower curve) The P D at F was brought nearly to zero by the
application of 0 01 M KCl before the record started All contacts were Solution A
in which the cell had been kept for 5 days The vertical lines represent 5 second
intervals Room temperature 22 C

0 001 м KCl at 1°C was applied at D (middle curve), causing a negative variation which spread in both directions (the action curve with a single peak is characteristic of *Chara*) It will be observed in Figs 8 and 9 that the polarization of the cell was only partly dis-
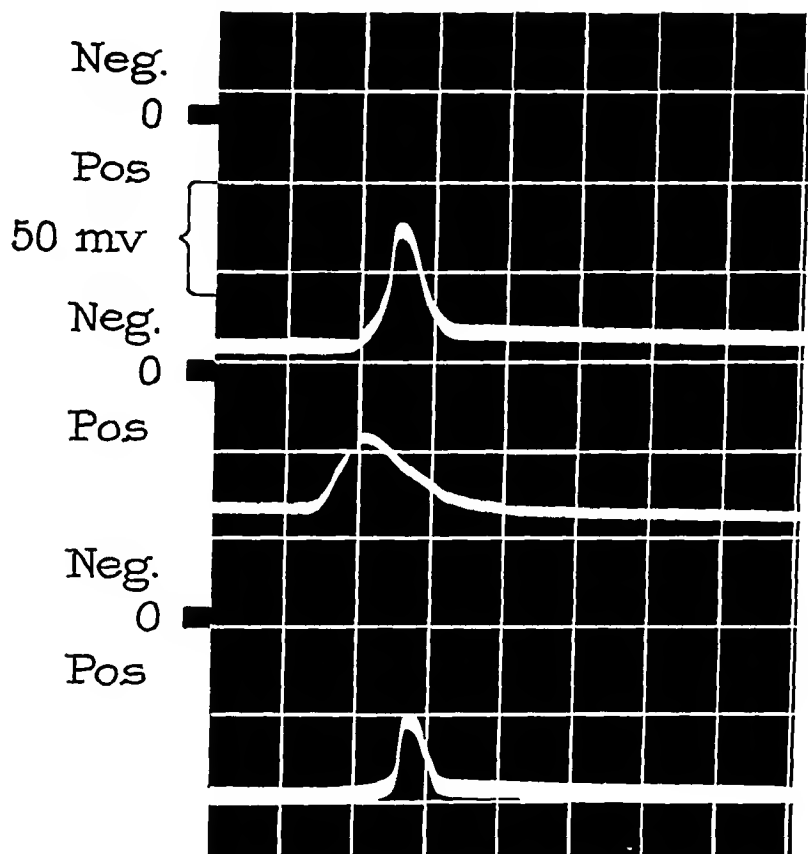
FIG 8 Photographic record of negative variations in *Chara* caused by the application of 0 001 м KCl at 1°C at D (Fig 4) The top curve shows the changes in potential at C, the middle curve at D, and the bottom curve at E All contacts were 0 001 м KCl F was killed before the experiment started The cell was kept for 5 days before use in Solution A The vertical lines represent 5 second intervals Room temperature, 22°C

charged (*i c* the action curve does not go to zero) in these two experiments It is interesting that the phenomenon of stimulation of single cells by cold solutions could be observed in two genera of plants

It may be noted that solutions at 14°C rarely stimulate *Nitella* those at 10°C stimulate in about half the cases, those at 8°C stimulate in about 80 per cent of the cases, and those at 1°C in about 94 per cent
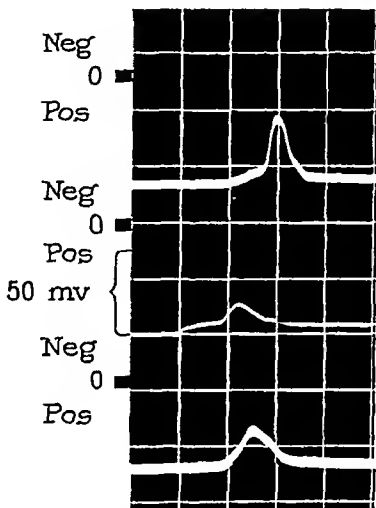
FIG 9 Photographic record of negative variations in *Chara* caused by the application of 0 001 M KCl at 1 C at D (Fig 4)   The top curve shows the changes in potential at C the middle curve at D and the bottom curve at E   All contacts were 0 001 M KCl   F was killed before the experiment started   The cell was kept for 5 days before use in Solution A   The vertical lines represent 5 second intervals   Room temperature 22 C

It appears possible that the action current starts as the result of a sudden break in the non aqueous surface layers of the protoplasm [14] At room temperatures we suppose these layers to be liquid but if they

[14] For a discussion of such layers see Osterhout W J V   *Ergebn Physiol* 1933, 35, 1021

were partly or wholly solidified by a fall of temperature[15] they might possibly be ruptured by the protoplasmic movement which is constantly present in these cells and which continues for 10 seconds to
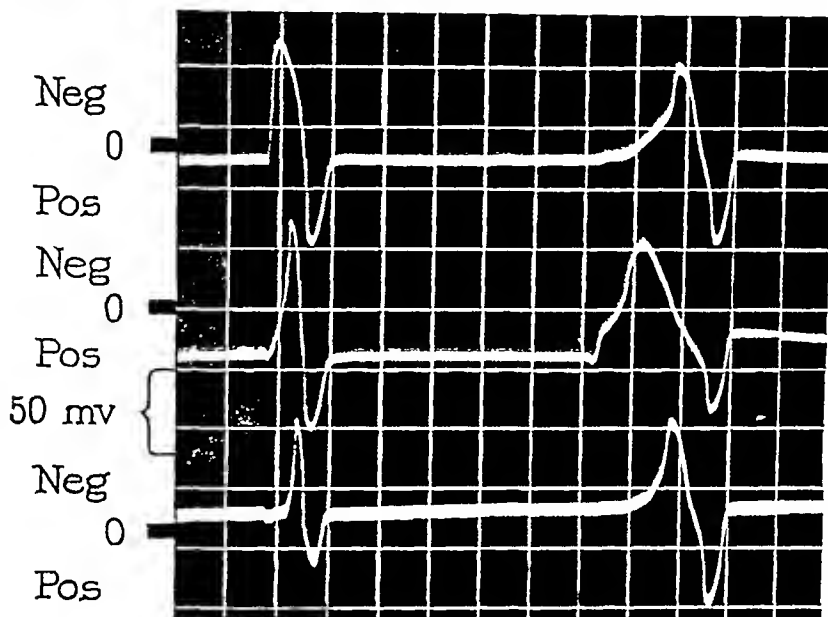
Fig 10 Photographic record of negative variations in *Chara*   All contacts were tap water, in which the cell was kept for 2 days before use   The first variation was caused by stimulation with 120 mv D C at *B* (Fig 4)   The negative variation passed along the cell, being recorded at *C* (top curve), *D* (middle curve), and *E* (bottom curve)   The second negative variation was caused by the application of tap water at 1°C at *D* (Fig 4)   This negative variation passed along the cell in both directions, being recorded at *C* and *E*

The simultaneous downward movement of the three curves (diphasic response) is caused by the response at *F*, the common contact

The vertical lines represent 5 second intervals   Room temperature, 21°C

several minutes after the start of the action current set up by the cooling   The writer has found that a spot in contact with Solution A at 1°C is much more sensitive to mechanical stimulation than at

[15] As the temperature is lowered certain oils show sudden and great increases in viscosity (*cf* Gasser, H S , *Am J Physiol* , 1931, **97**, 254)   If the non-aqueous protoplasmic surface behaved in this way it might easily be ruptured below the critical temperature

room temperature The outer layer λ would seem to be less susceptible to rupture when chilled, owing to its adherence to the cellulose wall and its distance from the actively streaming protoplasm When the action curves seen at the chilled spot show a double peak[16] this can be accounted for if we suppose λ to remain intact when Y is ruptured

Slow cooling does not appear to produce action currents This might be due to the fact that protoplasmic streaming falls off so rapidly that it can no longer rupture the protoplasmic surface at the time when the latter has become solidified Other changes may occur on cooling which contribute to this result

Chilling to $10°C$ or lower may cause a block so that a negative variation may be unable to pass the chilled spot This may be due to the fact that the chilled and partly solidified non aqueous surface layers are incapable of the sudden increase of permeability (due to electrical changes) which is apparently characteristic of the negative variation [16]

It may be added that the salting out ratio alters with temperature [17] A change of temperature could therefore cause changes of potential by causing salts to move in or out of the non aqueous protoplasmic surfaces which are the chief seats of $E M F$ Whether this has any effect in this case is uncertain

SUMMARY

Sudden local chilling causes action currents to be set up in *Nitella* and in *Chara*, an effect which does not follow gradual local chilling This may be due to a partial solidification of the non aqueous protoplasmic surfaces which makes them susceptible to rupture by the protoplasmic streaming This movement continues usually for several minutes after the chilling whereas if stimulation occurs at all it occurs immediately on chilling It is found that a chilled spot is much more sensitive to mechanical stimulation than is a spot at room temperature

Chilling is accompanied by a rise of resistance a lowered rate of recovery following stimulation, and usually by a falling off in the magnitude of the action curve

[16] Osterhout W J V *J Gen Physiol* 1934-35 **18** 215
[17] Randall M and Farley C T *Chem Rev* 1927 **4**, 291

POSITIVE VARIATIONS IN NITELLA

By W. J. V. OSTERHOUT AND S. E. HILL

(From the Laboratories of The Rockefeller Institute for Medical Research)

Under suitable conditions we find positive and negative variations in the same cell of *Nitella*. As this is of some theoretical interest a brief account of the experiments is here presented.

By way of definition we may say that when stimulation produces a temporary loss of P D at a spot which is positive[1] in its resting state the electrical variation is called negative (all the variations described in the literature are of this kind). It follows that when stimulation produces a temporary loss of P D at a spot which is negative in the resting state (so that the spot becomes less negative) the electrical variation may be designated as positive.

In some earlier experiments[2] cells were stimulated mechanically by cutting. The cut created a compression wave which traveled along the cell, causing a loss of P D at every point. A spot which was in contact with 0 001 M KCl and therefore positive, became more negative. A spot in contact with 0 1 M KCl, and therefore negative, became more positive. For example, two spots, A and C, were made negative by applying 0 1 M KCl. The cell was then cut close to A. This caused the P D to fall to zero[3] more rapidly at A than at C.[4] In consequence A became temporarily positive to C.[5]

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[1] The P D is called positive when the positive current tends to flow from the sap across the protoplasm to the external solution.

Historically the term *negative* was applied to the electrical variation not because it becomes more negative (when the potential is defined in the conventional way) but because a loss is involved. By usage however a negative variation has come to mean a change by which a spot becomes more negative and we shall so consider it.

[2] Osterhout W J V and Harris E S *J Gen Physiol* 1928-29 **12**, 167 355
Osterhout W J V and Hill S E *J Gen Physiol* 1930-31 **14**, 385 473

[3] Cf Osterhout W J V and Harris E S *J Gen Physiol* 1928-29 **12**, 167

[4] The process begins almost simultaneously at both places but proceeds more rapidly at 1 because it is nearer to the cut

[5] Cases have also been described (cf Osterhout W J V and Harris E S

This positive variation is not completely analogous to a negative variation since for one thing it is not reversible   But we may stimulate reversibly by pinching or bending the cell instead of cutting it We thus obtain positive variations which are reversible

This may be illustrated by some typical experiments   The record in Fig 1 was made by arranging two cells[6] as in Fig 2 with a nutrient
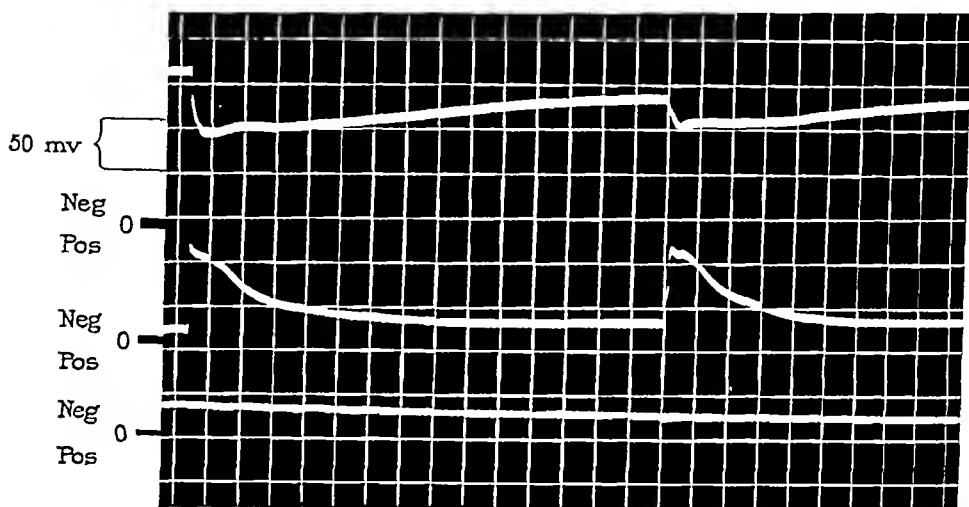
FIG 1 Photographic record of experiment arranged as in Fig 2   Room temperature 21°C   The vertical marks are 5 seconds apart   The electrical variations followed a pinch near contact A The top curve records changes at A, the middle curve changes at B, and the bottom curve shows that little change occurred in the circuit connecting C and D Upward deflection of the string represents relative increase in negativity of A, B, or D Any change of potential at C would result in simultaneous movements in the same direction of all three curves, and any change at the junction of the two cells would result in simultaneous movements in the same direction of the two upper curves   (No such change occurs in this case) A was in contact with 0 1 M KCl + 0 01 M CaCl₂, and B, C, and D were in contact with Solution A,[7] in which the cell had been kept for 10 days

Gen Physiol , 1927–28, 11, 695 (Fig 18)) in which chloroform was applied to a spot in contact with 0 1 M KCl, as the result of which the spot became less negative, then more negative, and then went to zero   This might be interpreted as a positive variation superimposed on a death curve

[6] The plant is Nitella flexilis, Ag   The technique, unless otherwise stated, is that described in previous papers (Osterhout, W J V , and Hill, S E , J Gen Physiol , 1930–31, 14, 473, 1933–34, 17, 87)

[7] For the composition of this solution see Osterhout, W J V , and Hill, S E , J Gen Physiol , 1933–34, 17, 87   Its effect on the P D is approximately that of 0 001 M KCl

solution (Solution A[7]) at B, C, and D A was in contact with 0 1 M KCl (containing 0 01 M CaCl₂ for purposes of physiological balance) which made the P D negative[8] The P D at C was positive and A was 148 mv negative to C as seen in the top curve which shows the P D of A with reference to C

When the cell was stimulated mechanically by pinching near A there was a loss of P D at A and B (just as when the stimulus was pro
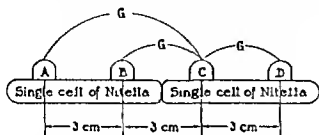
Fig 2 Arrangement used in making the record shown in Fig 1 on two naturally united cells from the same filament of *Nitella* Contacts B C and D are Solu tion A and contact 1 is 0 1 M KCl + 0 01 M CaCl₂ The cells were placed on a paraffin block in a moist chamber and the contacts made through calomel elec trodes GGG represent string galvanometers with thermionic amplifiers (all three strings in the same magnetic field) The method of measurement is es sentially electrostatic
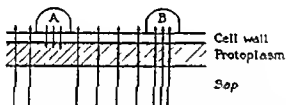
Fig 3 Hypothetical diagram of the P D across the protoplasm of the cell whose record is shown in Fig 1 A is in contact with 0 1 M KCl and B with Solution A The arrows show the direction in which the positive current tends to flow and their length indicates very roughly the magnitude of the P D

duced by cutting as already described) But the loss was temporary, and the process was reversible Although A and B were 3 cm apart the start of the response was practically simultaneous at both places as would be expected since the compression wave produced by pinching travels along the cell at a high rate of speed[2]

[8] This has practically the same effect on the P D as 0 1 M KCl For the latter see Osterhout W J V and Harris I S *J Gen Physiol* 1927-28 **11**, 673 1929-30 **13**, 47

The temporary loss of P D at A produced a positive variation (as shown in the top curve) because A was negative but at B it produced a negative mechanical variation[9] (as exhibited in the middle curve, which shows the P D of B with reference to C) because B was positive

A second pinch near A produced a similar result

The bottom curve (showing the P D of C with reference to D) exhibits no change except a slow drift   This means that the mechanical stimulation did not pass[10] to the cell at the right   Hence it is evident that all the changes recorded in the top and middle curves (except the slow drift just mentioned) occurred at A and B

If the slow drift seen in the bottom curve occurred at C[11] it means that a correction should be applied to the P D of A as seen in the top curve   When this is done it is seen that the recovery after the second positive variation is practically complete, while that after the first positive variation is nearly complete

We may picture the situation in the cell as in Fig 3   When the cell is pinched at or near A, a compression wave travels along the cell [2][3][12]   On reaching A it causes a loss of P D (partial or complete) Perhaps the simplest assumption is that this is due to a mechanical rupture of the non-aqueous protoplasmic surface layers   Previous experiments indicate that the more violent the mechanical disturbance

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[9] Such variations are called mechanical to distinguish them from ordinary negative variations not produced by mechanical stimulation   Some points of difference have been discussed in a previous paper (Osterhout, W J V, and Hill, S E, *J Gen Physiol*, 1930–31, **14**, 473)   Others appear on comparing the curves given here (and in the previous paper) with the figures showing the ordinary propagated negative variation (as shown in previous papers), but as these figures do not show the great variety of forms which occur in the two kinds of variation caution must be used in drawing conclusions in this respect

[10] Inasmuch as the circuit from A to C passes through the end of the left-hand cell a disturbance at that spot might be registered   An ordinary negative (non-mechanical) variation does not as a rule affect the bottom curve when the cells are arranged as in Fig 2   Cf Osterhout, W J V, and Hill, S E, *J Gen Physiol*, 1929–30, **13**, 547

[11] If the slow drift occurred at D it would show in the bottom curve but changes at D would not show in the top or middle curve since in these cases D was not in the circuit

[12] Osterhout, W J V, and Hill, S E, *J Gen Physiol*, 1930–31, **14**, 473

the greater the loss[13] of P D (as the compression wave travels along the cell its effects diminish) and the greater the danger of irreversible injury Repeated pinching is apt to cause injury Fig 4 shows that after the first four pinches recovery was complete but not after the fifth and sixth

The fact that the loss of P D is gradual may be due to a series of ruptures followed by immediate repair in the fashion of a contractile vacuole discharging itself by a rupture of the surface[14] The loss of
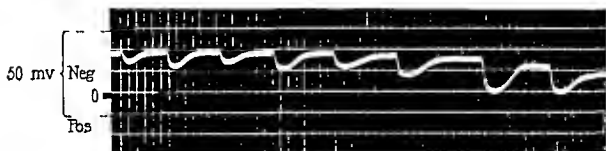
FIG 4 Photographic record of experiment arranged as in Fig 5 Room temperature 25 C The vertical marks are 5 seconds apart The electrical variations were set up by bending near contact A Contact B was killed by chloroform before the record started and the movement of the string results wholly from change in potential at A Downward deflection of the curve shows that A is becoming more positive

P D would then depend on the number of ruptures existing at a given instant and might be gradual (this would also apply to recovery)

Since the outside concentration of K^+ is greater than that inside there may be some penetration of K^+ (due to the rupture or other changes taking place during the positive variation) in which case recovery would not be complete until the penetrating K^+ had diffused away into neighboring regions

When we consider Fig 3 we see how the compression wave acts When it causes a loss of P D at A this spot becomes more positive but

[13] Osterhout W J V and Harris E S J Gen Physiol 1928-29 12, 1/9 (Figs 16 a and 16 b) Osterhout W J V and Hill, S E J Gen Physiol 1930-31 14, 481 (Fig 11)

[14] The senior author has made artificial models which illustrate this perfectly In some of these models numerous ruptures occur simultaneously or in quick succession each being followed by repair

when it causes a loss of P D at B this spot becomes more negative (here too the loss may be partial or complete)

It is evident that the procedure is quite different from that in a propagated negative variation of the usual sort [12] According to the local circuit theory such a variation would not be able to pass A because it is propagated by setting up at successive spots on the cell an outgoing electrical current When it reached A it could not do this and in consequence A would act as a block This is confirmed by experiment
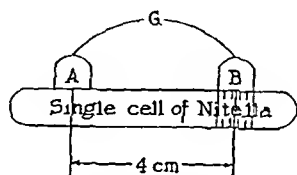
Fig 5 Arrangement used in making record shown in Fig 4 Contact A is 0 1 M KCl + 0 01 M CaCl₂, and contact B is Solution A + CHCl₃ The cell was kept in Solution A[7] for 10 days before use The cell was placed on a paraffin block in a moist chamber and contact made through calomel electrodes to G, representing a string galvanometer with thermionic amplifier The method of measurement is essentially electrostatic

It is likewise evident that a propagated negative variation could not pass a killed spot but that a compression wave might do so This is also confirmed by experiment [12]

An interesting experiment is recorded in Fig 4 which shows a monophasic record of eight positive variations set up by successively bending[15] the cell near A (the cell was arranged as in Fig 5) B was killed by means of chloroform so that it could exhibit no changes in potential A was in contact with 0 1 M KCl + 0 01 M CaCl₂ and was 24 mv negative to B as shown by the record (since the P D at B was practically zero the values shown in the record are those at A) The bending at A was carefully done, and the first four responses, while variable in extent, showed complete recovery [16] Recovery after the fifth and sixth stimuli was not complete The seventh and eighth

[15] The number of pinches which the cell can endure without injury depends on the magnitude of the mechanical disturbance and the state of the cell Sometimes a single pinch causes permanent injury

[16] It will be observed that the coordinates have been given values which emphasize the responses The sensitivity of the string, as compared with that in

stimuli, which sent the potential approximately to zero, were also followed by incomplete recovery

It is evident that the positive variations described in this paper are analogous to the negative mechanical variations described earlier and it therefore seems appropriate to call them positive mechanical variations

SUMMARY

The reversible electrical variations hitherto described for plants and animals consist in a reversible loss of positive potential at a stimulated spot by which it becomes more negative

In this paper we describe changes which consist in a reversible loss of negative potential at a stimulated spot whereby it becomes more positive   We suggest that this be called a positive variation

The stimulation was produced in all cases by pinching or bending the cell   This produced a compression wave which traveled along the cell, producing a negative variation at a spot which was positive and a positive variation at a spot which was negative (due to application of 0 1 M KCl)

The response produced by the compression wave differs in several respects from an ordinary propagated negative variation and may be termed a positive mechanical variation

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Fig 1 is a little more than four times as great while the 5 second marks are only one half as far apart

NATURE OF THE ACTION CURRENT IN NITELLA

II. SPECIAL CASES

BY S. E. HILL AND W. J. V. OSTERHOUT

(From the Laboratories of The Rockefeller Institute for Medical Research)

In the first paper of this series the general features of the action curve of *Nitella* were interpreted as due to the movement of potassium ions accompanied by an increase in permeability.

Can this idea explain the variations which the action curve exhibits? For this purpose we may consider the successive movements. We may begin with a fairly typical action curve[1] such as is shown in Fig. 1. This was obtained by leading off as shown in Fig. 2 (the stimulus consisting of an outgoing electrical current at B). In this, as in all other records shown in this paper, the common contact F was killed with chloroform (unless otherwise stated), making the records monophasic. Inasmuch as curves like those shown in this paper have been obtained with cells in which no spot had been killed or injured it is evident that these forms of the action curve are not influenced by the killing of F.

The first movement (o, Fig. 1). If this is due to the passage of K^+ across the inner protoplasmic surface I, causing a loss of P.D. its speed and extent will depend on the rate of movement of K^+ and on the uniformity of behavior of the region which is being recorded. If at one spot K^+ begins to move rapidly the start of the o movement will show corresponding speed but the latter part of the movement may be slow because the process will be incomplete until I reaches the same state over the whole region involved. Before this happens K^+ may reach the outer non aqueous layer in some part of the region

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[1] We regard as fairly typical an action curve in which both the first and second peaks approach zero but there is a good deal of variation in this respect.

Very frequently we lead off from an area of cell 1 cm. in length all of which is in contact with the solution.

so that a positive potential may be built up at X which will prevent the o movement of the curve from reaching zero (such a positive potential may exist from the start this will be discussed in a later paper)   Furthermore, if K^+ moves so rapidly[3] across W that its concentration at the outer surface of Y does not reach the same level as at the inner surface the o movement may not go to zero[4] The curve sometimes lacks a good deal of going to zero as shown in Fig 3, but in general the approach to zero is closer than this[5]
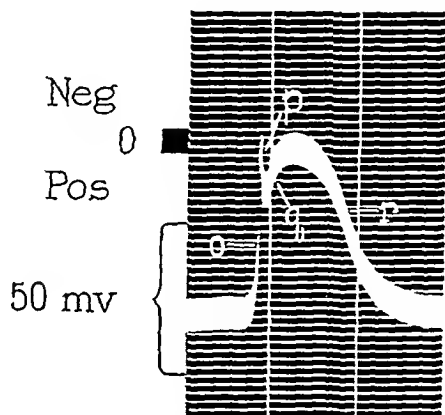
Fig 1 Photographic record showing a fairly typical action curve in which the o and q movements go to zero   The experiment was arranged as in Fig 2, employing contacts C and F only F was killed with $CHCl_3$ before the record started to secure monophasic recording at C C was in contact with 0 001 M KCl The cell had been kept in tap water before use   The vertical marks are 5 seconds apart   Temperature, 23°C

When the positive P D in the resting state depends in part on a positive P D across the outer protoplasmic surface, X, the o movement will not go to zero since the outward movement of K (which

[3] The time required for the movement varies according to the thickness of the layers and the degree of stirring produced by the protoplasmic motion (which is always present in *Nitella*)

[4] When the action curve has a single peak and the o movement does not go to zero the cause may be different   This will be discussed in a subsequent paper

[5] When we record the P D of a spot A with reference to another spot B in contact with chloroform the action curve of A sometimes appears to go beyond zero and become negative, but this is regarded as due not to negativity at A but to positivity at B

causes the loss of the P D across Y) will not cause the P D across λ to disappear at once   As K^+ moves on across W to λ it will increase the positive P D across Y, thus causing the p movement   When it reaches the outside of λ it may cause the P D across λ to disappear, thus carrying the q movement to zero, or nearly to zero, as in Fig 3

As previously explained[6] there may be a breakdown (partial or complete) in the I layer which might cause the o movement quite independently of any migration of K^+

The second movement (p, Fig 1)   If K^+ traveled outward in the form of a sharply defined moving boundary we should expect a sudden increase of positive P D when it reached the inner surface of λ and this might carry the p movement back to the starting point of the
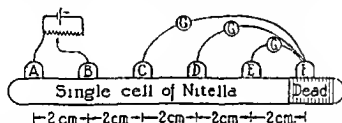
Fig 2   Arrangement for testing *Nitella* cells   GCC represent string galvanometers with vacuum tube amplifiers arranged essentially as short period electrostatic voltmeters   For details see Osterhout W J V and Hill S E   *J Gen Physiol* 1933–34 **17**, 87   Absorbent cotton moistened with the contact solution connected the cells to saturated calomel electrodes between contacts the cell was surrounded by air

action curve if K^+ produced as much effect on λ as on I This seldom happens but an instance in which it goes down a good way is shown[7] in Fig 4 and one in which it goes back to the starting point is seen in Fig 5

The fact that the p movement does not usually descend very far is not surprising as the moving boundary tends to lose its sharpness in passing across W since this is a comparatively thick aqueous layer[8] which, in *Nitella*, is continually agitated by protoplasmic motion [9]

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[6] Osterhout W J V   *J Gen Physiol* 1934–35 **18**, 215

[7] In these cases the thickness of the protoplasmic layer II and the protoplasmic motion in this layer were probably smaller than usual

[8] The total thickness of the protoplasmic layer is not much more than 10μ the layers λ and I may be very thin

[9] This may be true in lesser degree of I *alone*
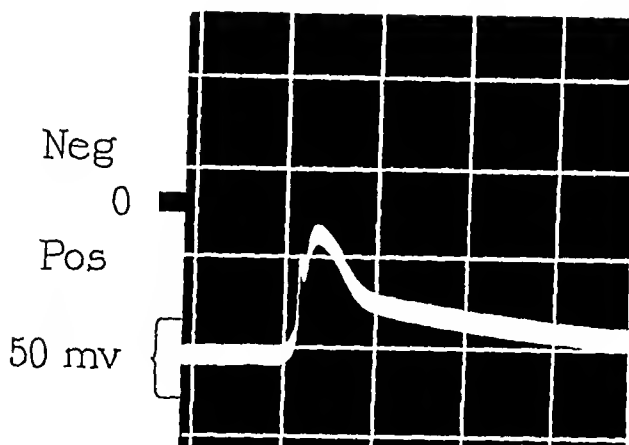
FIG 3 Photographic record showing an o movement which does not go to zero The record was arranged as in Fig 2, employing contacts C and F only F was killed with ethyl alcohol before the record started to secure monophasic recording at C C was in contact with 0 001 M KCl The cell had been stored in Solution A (for the composition of this see Osterhout, W J V, and Hill, S E, J Gen Physiol, 1933-34, 17, 87) for 23 days and in 0 001 M NaOH for 1 hour before use The vertical marks are 5 seconds apart Temperature, 22°C
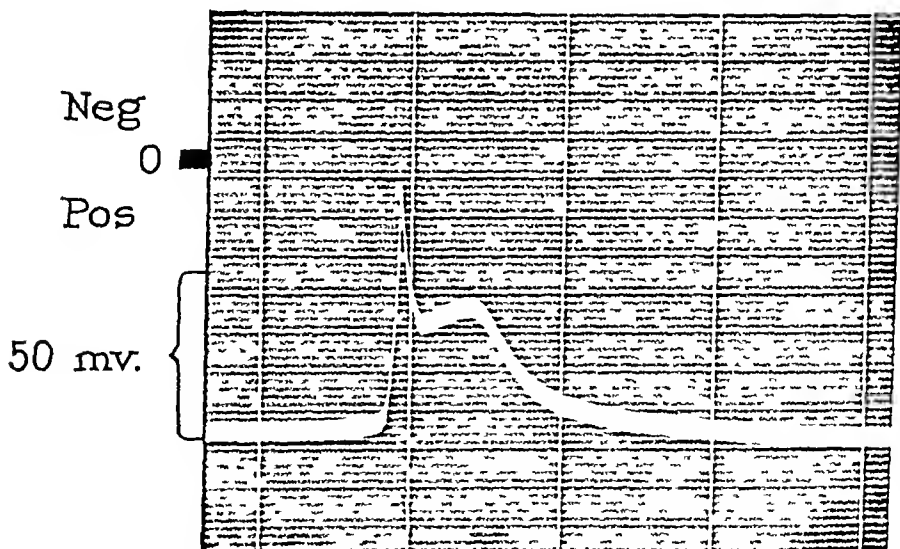
FIG 4 Photographic record in which the p movement is greater than usual and the q movement less than usual The experiment was arranged as in Fig 2, employing contacts C and F only F was killed with CHCl₃ before the record started to secure monophasic recording at C C was in contact with Solution A, in which the cell had been kept for 10 days The vertical marks are 5 seconds apart Temperature, 20°C

In consequence the concentration at the inner surface of V would not as a rule reach the value found at the inner surface of J (especially as it would be lessened by outward diffusion through V)   The same factors
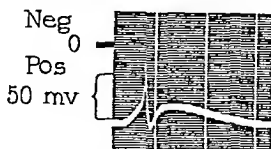
Fig 5 Photographic record in which the p movement descends to the starting point of the action curve The experiment was arranged as shown in Fig 2 employing contacts C and Γ only Γ was killed with $CHCl_3$ before the record started to secure monophasic recording at C C was in contact with Solution A in which the cell had been kept for 10 days The vertical marks are 5 seconds apart Temperature 20 C
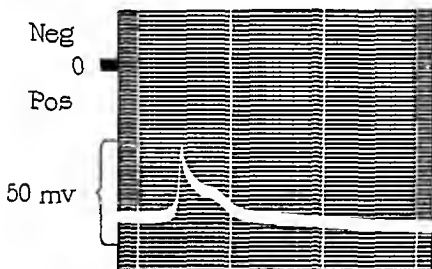
Fig 6 Photographic record showing the q movement much reduced The experiment was arranged as shown in Fig 2 employing contacts C and Γ only Γ was killed with $CHCl_3$ before the record started to secure monophasic recording at C C was in contact with 0 001 M KCl The cell had been kept in Solution V for 13 days before use The vertical marks are 5 seconds apart Temperature 20 C This figure recalls some of the records obtained by Casser with nerve

would tend to make the slope of the curve less than in the o movement [10]

[10] Lack of uniformity in the various parts of the region recorded would also diminish the rapidity since the movement would not be finished until all of the region responded

The third movement (q, Fig 1)   This is presumably due to the continued outward movement of K which increases the concentration of K$^+$ just outside X This process would ordinarily be slow since K$^+$ would be carried away by convection currents in the film of solution between the protoplasm and the cellulose wall as well as by diffusion through the cellulose wall (which is very rapid) [11]   The final level of concentration (which determines the height reached by the q movement) depends on how much K$^+$ reaches the outside of X before the process of recovery begins so that it may be expected to show considerable variation   This is actually the case (Figs 1, 3 to 6)

The fourth movement (r, Fig 1)   This movement involves the return of the cell to the resting state in which the concentration of potassium in W is small   This can no doubt be brought about by those forces which in the resting state of the cell cause potassium to move from the external solution to the sap and accumulate there   Such forces could move potassium from W into the sap until the original resting state was achieved   This may possibly be hastened by the ingoing electrical current which, according to the local circuit theory, occurs during recovery   Recovery is, however, a relatively slow and variable process   Normally it takes about 15 seconds but it may be shorter and it is sometimes greatly prolonged

When recovery remains permanently incomplete the protoplasm is regarded as no longer in a normal state

Some of the curves obtained with *Nitella* resemble those gotten with nerve   For example, curves like that in Fig 6 somewhat resemble those described by Gasser [12]   If such records were obtained with a single nerve fibre it might raise the question whether there may possibly be two surfaces, X and Y, in nerve

If this assumption were made it might explain the form of the action curve and also the current of injury which is otherwise hard to account for   If the nerve fibre consisted wholly of protoplasm, cutting it might

[11] When 0 01 M KCl is substituted for 0 001 M the change of P D is nearly complete in 5 seconds, showing that diffusion through the cell wall is very rapid cf Osterhout W J V *J Gen Physiol* 1929–30, **13**, 715

[12] Gasser, H S *Am J Physiol*, 1931, **97**, 254, Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, Long Island Biological Association, 1933, **1**, 138

automatically give rise to a fresh protoplasmic surface which would have the same electrical properties as the normal surface [13] In that case there would be no current of injury But if any part of the core of the nerve fibre were non living and ran continuously lengthwise as in *Nitella* the current of injury could be explained in the same way as in *Nitella*, for in spite of the fact that the cut surfaces of the pro toplasm healed at once the circuit would pass only once through living protoplasm

The forms of the action current discussed in this paper (except Fig 1) are relatively infrequent and constitute less than 5 per cent of the action curves observed by us

SUMMARY

The action curve involves four movements each of which shows considerable variation These variations can be accounted for on the assumption that the action curve is due to the movement of potassium ions accompanied by an increase in permeability

[13] This is found experimentally in such cells as *Ameba* and is to be expected if the ordinary surface is formed by substances which migrate into it because they are surface active (Osterhout, W J V *Ergebn Physiol* 1933 **35**, 1020)

THE LIGHT GROWTH RESPONSE AND THE GROWTH SYSTEM OF PHYCOMYCES

By E S CASTLE AND A J M HONEYMAN

(*From the Laboratory of General Physiology, Harvard University, Cambridge*)

I

The spore bearing cell of *Phycomyces* has a growing zone at its distal end which is sensitive to light   An increase in illumination causes a temporary increase in rate of elongation of the cell, the so called light growth response   Light has this effect only if it strikes the growing zone, and the response is produced at the same place   This region of the cell therefore comprises the functions of a "single receptor" and "single effector "   The most valuable measure of the response to light is the reaction time,[1] which may occupy from 2 to 10 minutes, and is almost entirely latency, since the time of exposure to light need only be a fraction of a second   In multicellular plants the reaction times to light are longer, due to the transmission of hormonal effects   Nothing of this kind is known for the coenocytic cell of *Phycomyces*, yet the time elapsing between stimulation and response is a matter of minutes   This latency is comparable to the lag which occurs in a typical animal photoreceptor between the photochemical effect and the discharge of impulses in the attached nerve, except that it is several thousand times longer   In the present paper an attempt is made to interpret this exceptionally long latency in terms of the structure of the cell of *Phycomyces* and its mechanism of growth   Information is almost entirely lacking for cells of any type as to what kind of events translate the light effect into the response

II

Data have been obtained with *Phycomyces* relating reaction time and duration of exposure to light for several different intensities

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[1] Defined as the time from the beginning of stimulation to the first perceptible acceleration of growth

385

The conditions of the experiment were as shown in Fig 1   The data are given in Table I, the mean reaction times are plotted in Fig 2 Inspection of the curves shows (1) that as the length of exposure to light increases, the reaction time shortens to a minimal value determined by the intensity, (2) longer exposures do not further shorten the reaction time, (3) the range of exposures which is effective in shortening the reaction time appears to be shorter the higher the intensity of light

The significance of these measurements is best seen if quantities of light (intensity × time) are plotted on the abscissa instead of ex-
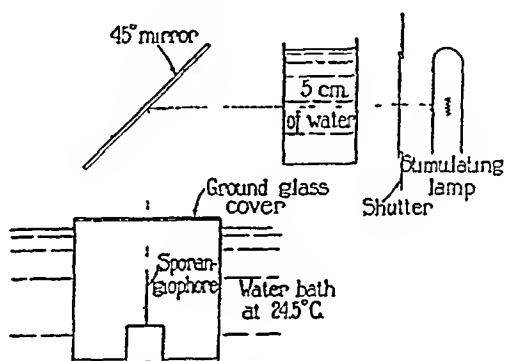
FIG 1 Diagram of experimental conditions   From a source not shown in the figure, the culture was continuously illuminated from above throughout the experiment with 0 0017 foot-candles, except during the brief stimulating exposure This served to orient the growth of the cells at all times   Different exposures were obtained with a calibrated photographic shutter   Intensities were measured photometrically at the level of the sporangiophore

posure times   (See Fig 3) The upper portions of all the curves are brought together, showing that (within a certain range) to produce a response after a particular reaction time a constant quantity of light is needed, irrespective of its time and intensity components   This relation is the familiar Roscoe-Bunsen law   Of more interest is the fact that the data show the failure of this relation to hold when the time component in stimulation becomes large   Thus in Fig 2 each curve falls toward a base line determined by the intensity alone, and not by the energy of the flash of light   In Fig 3 the curves begin to separate at an energy of less than 10 foot-candle seconds   Something limits the *effective* duration of the exposure to light   That this

is not simply due to the using up of all of the photosensitive material
is shown by the fact that the curves for the different intensities reach
different hase lines   Evidently secondary processes follow the photo
chemical reaction, and get under way with velocities determined by
the light intensity

Families of curves similar to those of Fig 2 were obtained by Hecht
(1918) for the latent period of the light response of the clam *Mya*, and
by Adrian and Matthews (1927) for the retinal latent period of the
eel's eye   The data of Hartline (1928) on the magnitude of the
electric response of the grasshopper's eye yield comparable curves in
which for each intensity the potential which is developed rises with
increasing exposure toward a limiting value determined by the in
tensity of the light   In all these cases and in the present one, light
acts on a photochemical system which is coupled to secondary proc-
esses leading to specifically different responses   The dependence of
the reaction time of *Phycomyces* on intensity shows that light does not
promptly pull a trigger   Not only is the ultimate growth response
graded, but the time taken to bring it about is determined by the
stimulating intensity

III

The response of *Phycomyces* to light is remarkable in that after ex-
posure to even very intense illumination there is a latency of at least
2 minutes before any change in rate of growth is observed   When
the acceleration does occur, the growth curve rises sharply   (See
Fig 4)   What goes on during this long latent period?

Light accelerates the growth of *Phycomyces* but at this stage of
development is not necessary for it   Over a sufficient period of time,
the total elongation is independent of the presence of light or the oc
currence of light growth responses   The plots in Fig 4 show that
after a temporary acceleration induced by a flash of light, the course
of growth gradually returns to where it would have been had there
been no flash of light   Exactly the same holds for the dark growth
response   on darkening there is a temporary decrease in growth rate
which is completely compensated for when the light is turned on again
Light must act therefore by facilitating changes already under way,

TABLE I

Reaction Times with Different Intensities and Durations of Exposure to Light

Intensity	Exposure	Intensity × exposure	Reaction times	Average $R\,T$
foot candles	*sec*	*foot-candle sec*		*min*
89 3	6 0	536 0	3 25, 2 75, 3 50, 3 25, 3 25 3 50, 3 50, 3 75, 3 50, 3 25	3 35
	2 0	179 0	3 50, 3 25, 3 25, 3 00, 4 25 3 75, 3 00, 3 00, 3 50, 3 25	3 38
	0 60	53 6	3 75, 3 75, 3 75, 3 75, 3 00 3 75, 2 75, 3 25, 3 50, 3 50 4 25	3 55
	0 25	22 3	3 75, 4 25, 4 50, 4 00, 3 50 4 50, 4 00, 4 00, 3 75, 3 75	4 00
	0 08	7 14	4 50, 4 50, 4 50, 4 50, 4 75 4 75, 4 50, 4 25, 4 50, 4 50 4 50	4 57
	0 01	0 89	6 00, 5 25, 5 75, 5 50, 5 25 5 75, 5 50, 5 00, 5 50, 4 75	5 43
11 0	10 0	110 0	3 75, 3 75, 4 00, 4 25, 4 50 4 00, 4 00, 3 75, 3 50, 4 00	3 95
	3 0	33 0	3 75, 4 50, 3 75, 4 25, 4 00 4 00, 4 50, 3 75, 4 25, 4 00	4 08
	2 0	22 0	4 75, 4 25, 4 50, 4 00, 4 00 4 50, 4 00, 3 75, 4 50, 4 50	4 28
	1 0	11 0	4 75, 4 75, 4 50, 4 50, 4 25 4 25, 4 75, 4 50, 4 50, 4 50	4 53
	0 60	6 60	5 00, 4 75, 4 25, 4 50, 5 00 5 00, 4 50, 4 75, 4 75, 4 75 4 75	4 73
	0 25	2 75	5 25, 5 00, 5 75, 5 25, 5 00 5 25, 5 25, 5 25, 5 50, 4 75	5 23
	0 08	0 88	5 25, 5 00, 5 75, 6 00, 5 75 6 25, 6 25, 6 25, 5 25, 5 00	5 68

TABLE I—*Concluded*

Intensity	Exposure	Intensity × exposure	Reaction times		Average R.T
foot-candles	*sec*	*foot-candle sec*			*min*
1 22	3 0	3 66	5 00 5 25 5 25 5 25 5 00		
			4 50 5 00 4 50 4 50 4 75		4 90
	2 0	2 44	5 25 5 25 4 75 5 25 4 75		
			4 75 5 25 4 25 4 50 5 75		4 98
	1 0	1 22	4 50 5 00 5 75 5 25 5 00		
			5 50 5 25, 5 50 5 75 5 00		5 25
	0 60	0 73	6 75 6 75 5 75 6 00 5 50		
			5 00 5 50 5 75 5 75 6 00		
			5 75 5 25		5 81
	0 36	0 44	6 00 6 00, 6 00 6 00 6 00		
			6 25 6 25 6 25 6 25 5 75		6 08
0 08	4 0	0 32	4 75 6 25 4 75 5 75 5 75		
			5 75, 5 25 5 50 5 75 5 75		5 53
	0 60	0 05	6 00 6 25 7 50 7 25 7 00		
			6 25, 6 25 6 25 7 00 7 50		6 73

rather than by contributing essential materials [2] The accelerator which it produces is to be contrasted with auxin, without which growth of *Avena* coleoptiles cannot occur (Went, 1928)

Oort s (1932) measurements were not continued quite long enough to show that no significant increment in length is added by the response to a flash of light This statement does not refer to effects of light or darkness maintained for long periods of time Thus the sporangiophore of *Phycomyces* reaches a final length which is greater the lower the intensity of light in which it is grown This effect bears no close relation to the growth response induced by sudden exposure to light

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[2] This idea has not been tested in prolonged absence of light because of the difficulty of securing oriented measurable growth under these circumstances If it could be shown that the action of light really produced a material essential for growth it would still be true that over an enormous range of intensities light is not a limiting factor

The latent period might be taken up by one of the following events

1 A remote step in the growth process is accelerated, the latency is the time for this acceleration to pass down the sequence of growth processes to the final stage, the extension of the cell wall

2 The cell protoplasm is stimulated to faster secretion of wall substance
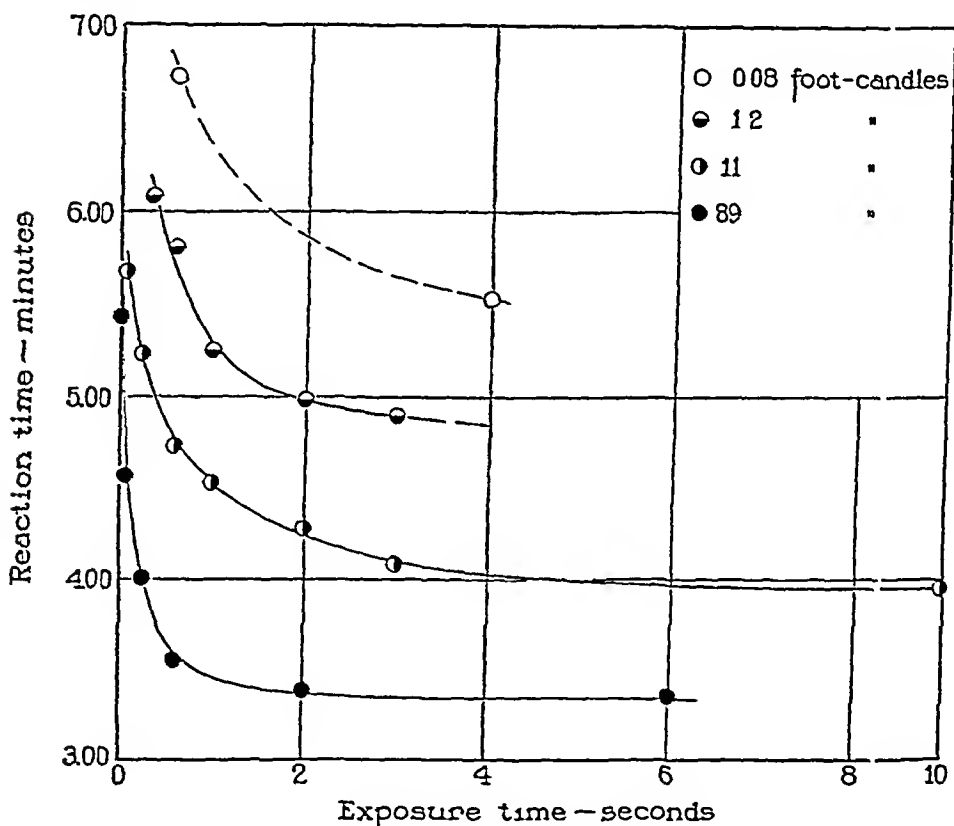
FIG 2   Mean reaction times with different intensities and durations of exposure to light.

3 A product of the action of light in the protoplasm diffuses or is transported to the cell wall

The first possibility is rendered improbable for the following reasons light does not act on a spatially remote stage of the growth process, since Blaauw (1918) has shown that the zones of growth and of sensitivity to light coincide, and extend less than 2 mm below the distal, spore-bearing end of the cell   If an average diameter of the cell is

0 09 mm , the whole system is localized in a volume not greater than $2\pi r = 0 013$ mm ³ The driving force behind the light growth re sponse is probably not thus localized, since if this force is turgor, all elastic strained parts of the coenocyte should contribute to the magni tude of the response   Furthermore, if light accelerated some early stage of the growth process localized within the growing zone we would
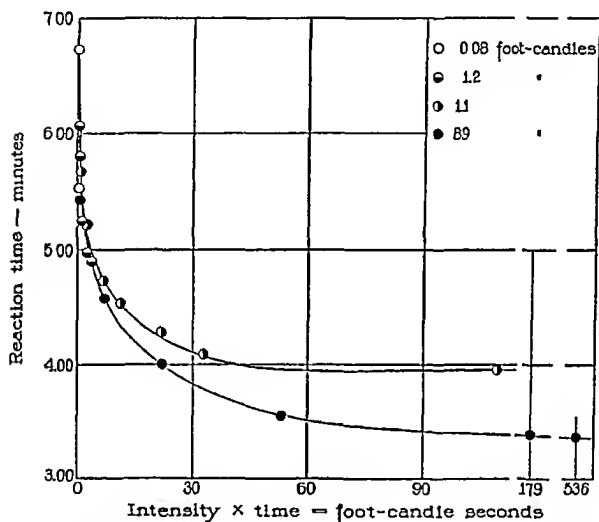
Fig 3 Mean reaction times to the different intensities plotted against the quantity of light (intensity × time)

expect fast growing cells to show shorter reaction times than slow growing ones, since the time required to propagate the effect through a chain of growth processes should be some inverse function of the rates of the processes   Fig 5 shows measurements of reaction times of cells growing at different rates under identical conditions of light, temperature, and sensitivity   There is no correlation of reaction time with rate of growth   This is evidence against the idea that the
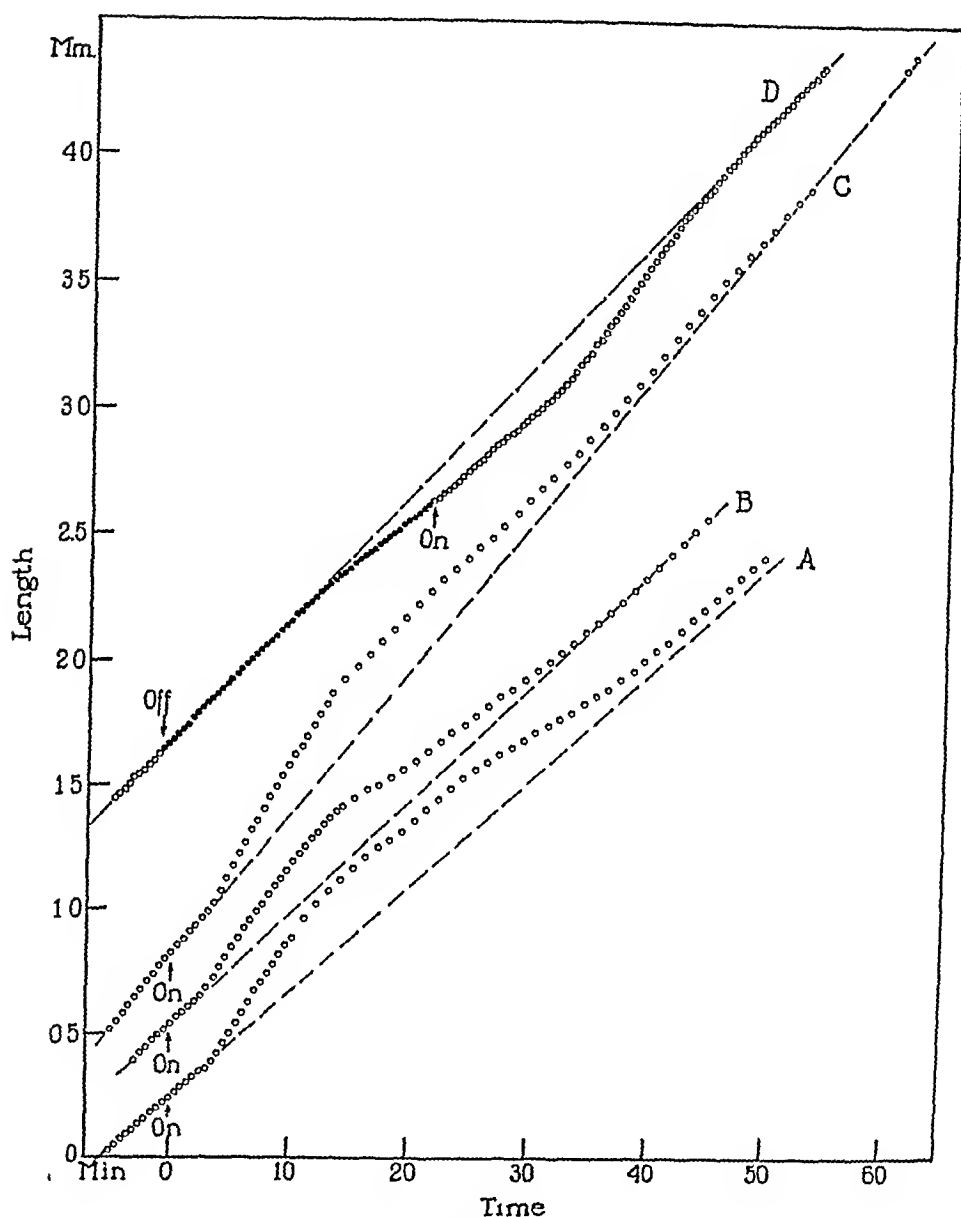
FIG 4 A, B, and C, typical light growth responses to 3 seconds intense stimulation ("On") from two sides   Note that within an hour the growth curve has approximately returned to where it would have been had there been no flash of light   In A there is a slight gain, in B and C none   Slight discrepancies in either direction may be expected with linear extrapolation of growth for such periods D, response to darkening during period "Off" to "On"   Note that the temporary loss in growth is completely regained when the light is turned on again   The ordinate and abscissa scales for D are twice those for the other curves

lag is due to the action of light on some antecedent stage of the growth process

The second possibility offers no separate explanation of the lag unless some meaning can be attached to the term "stimulation" Long latencies do not necessarily accompany stimulation by light, thus the latency in animal photoreceptors is usually less than 0 1 second While a slow "clock reaction," for instance of the type described by Forbes, Estill, and Walker (1922), might conceivably
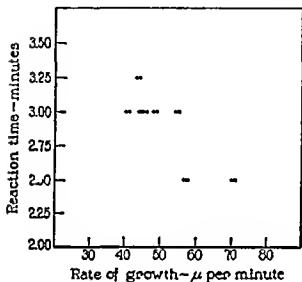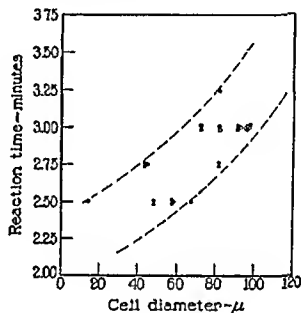
Fig 5 Individual reaction times of cells growing at different rates The grouping into definite ordinate classes is due to the estimation of the reaction time only to 1/4 minute intervals The data plotted here and in Fig 6 were obtained at 26°C as a result of 3 seconds very intense stimulation of the cells from two sides

Fig 6 Individual reaction times as a function of cell diameter Same cells as those in Fig 5

occupy the latent period, we prefer to seek a more tangible interpretation The relatively large size of the *Phycomyces* cell and particularly the relation to be described later between cell radius and reaction time suggest that radial translocation of the light effect is involved

In the third possibility the lag is due to time taken by some photolyte (or its derivative) in reaching the cell wall in sufficient amount There is no evidence permitting a final choice between the ideas (1)

that the ultimate action of light is on the physical properties of the cell wall, or (2) that the peripheral protoplasm is eventually stimulated to faster deposition of wall substance   The first alternative seems more probable, however, in view of the following the cell is a closed, incompressible system which is increasing in volume at a rate determined by processes taking place in the mycelium   These are uninfluenced by local illumination of the growing zone, several centimeters away   If, therefore, the cell suddenly gains in length it must be at the expense of a loss somewhere in diameter   In this sense the light growth response is a change in the *shape* of the cell rather than in its rate of volume increase   Such a change in shape is not a part of ordinary growth, and suggests a unidirectional change in the ductility of a wall substance, directly acted on by turgor

Consideration of the phototropic bending of the cell of *Phycomyces* has moreover suggested that absorption of light may take place throughout the cell, although the effect on growth is manifested at the wall[3] (Castle, 1933)   If during the latent period a sufficient quantity of some substance produced by light in the protoplasm of the cell had to be transported centrifugally to the wall, the radius of the cell might partly determine the latency   Measurements of individual reaction times of cells of different radii show that the larger cells have definitely longer reaction times   (See Fig 6) If all cells were uniformly filled with equal concentrations of light-sensitive substance, and if the response were set off by the accumulation of a definite amount of some photolyte per unit area of wall, the larger cells would have *shorter* reaction times, since in these cells the necessary amount of photolyte would be produced nearer the wall   The situation is evidently not as simple as this   There are at present no grounds for making other assumptions designed to fit the radius-reaction time data

Transmission of the light effect to the wall might take place by simple diffusion, by diffusion aided by protoplasmic streaming, or by some other conducting mechanism in the protoplasm   If reciprocals

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[3] Within the growing zone there is often no recognizable separation of the cell contents into protoplasm and sap vacuole   The lumen of the cell is filled with a granular, fluid mass in which no oriented protoplasmic streaming is evident (*cf* Oort and Roelofsen, 1932)   Sometimes a small central vacuole occupies 1/10 to 1/5 of the cell diameter

of reaction times are taken as approximate measures of the rates of the latent period process, the temperature coefficient for the interval 25°/15° is 1 7 But it cannot be concluded that this value is too high for diffusion since there is no assurance that the threshold of the growth process upon which the photolyte acts is unaffected by temperature change Furthermore, high values of temperature co efficient are often found for processes in which diffusion certainly is involved, as in the penetration of water into cells (cf McCutcheon and Lucké, 1932) Whether protoplasmic streaming, which is conspicuous except at the growing zone of the cell, is significant for growth is not known

The question as to the mechanism of transport of the light effect within the cell must be left open That centrifugal transport actually takes place seems inescapable (1) because the actual process of elonga tion occurs at the wall, and (2) because the latency is independent of all measured attributes of the cell except the radius It is note worthy that light is a stimulating agent that acts intracellularly A typical animal photoreceptor cell contains a pigment within it which absorbs light, then somewhat later a nerve impulse is set off The cell of *Phycomyces* is a good parallel on a larger scale and with a corre spondingly longer latent period In both cases the observed result of stimulation is an effect produced at the cell surface a nerve impulse or an extension of the cell wall Even structures as specifically dif ferent as those which are being compared may have an important step in common following the absorption of light in the transmission of excitation to the cell surface

IV

The relation between the light and growth systems in *Phycomyces* can be diagrammed as follows
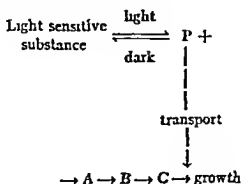
Above is shown the light system, with P the product of the action of light on a light-sensitive substance P is produced in the light and removed in darkness, and the direction of this change determines whether a light growth or dark growth response follows (*cf* Castle, 1932) P therefore exerts a continuous influence on growth even though it is not a limiting factor The light system is represented as reversible, which implies that P is not used up in growth The arrow labelled "transport" represents the direction of movement of P when light acts on the system When a steady state is reached there is presumed to be no concentration gradient of P

The growth system is represented below as a series of irreversible processes, one of which is facilitated by the presence of P (or a derivative of it) The latency in the response to light is due to the time needed for the transport of a sufficient quantity of P from its place of origin to the growth system, presumably to the cell wall or outermost layer of protoplasm

The light and growth systems are independent, in the sense that although a flash of light temporarily accelerates elongation, over a period of 1 to 2 hours any extra increment in length is lost, and the length of the cell becomes what it would have been had there been no flash of light Temporary darkening conversely does not result in a loss of growth over such a period of time

The facts of light and dark adaptation (Tollenaar and Blaauw, 1921, Castle, 1929) can be described in terms of the light system alone, as referable to changes in the concentration of light-sensitive substance present As judged by changes in the reaction time during dark adaptation, this process is practically complete within 40 minutes

Oort (1932) used the magnitude of the positive phase of the light growth response as a measure of sensitivity, and found that more than 2 hours must elapse after exposure to light before a second light response of full size was obtained His data show, however, that minimal reaction times were obtained after about 40 minutes of dark adaptation Therefore the light system must be restored after 40 minutes, and the extra $1\frac{1}{2}$ hours needed for further recovery represents the time necessary for the growth system proper to build itself up to equilibrium Although it is important to know what determines the size of the light growth response, use of its magnitude

as a criterion of sensitivity conceals the important fact that after a flash of light the light system reaches equilibrium in about one third the time required by the growth system

SUMMARY

1 The Roscoe Bunsen law holds for the light growth response of *Phycomyces* if the time component of stimulation is short   With exposures longer than a few seconds, the reaction time to light is determined by the intensity and not by the energy of the flash

2 The possible nature of the very long latency in the response to light is considered in terms of the structure of the cell and its mechanism of growth   It is suggested that during the latency some substance produced by light in the protoplasm is transported centrifugally to the cell wall or outermost layer of protoplasm

3 The total elongation occurring over a period of 1 to 2 hours is independent of flashes of light or temporary darkening   Light acts by facilitating some change already under way in the growth system, and during the principal phase of elongation is not a necessary or limiting factor for growth

4 Judged by the reaction time, the original sensitivity is restored in the light system following exposure to light in about one third the time required for equilibrium to be reattained in the growth system

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SOME TEMPERATURE CHARACTERISTICS IN MAN

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I

General diathermy (hyperpyrexia) treatments have been given by one of us (Perkins, 1931) to patients at the Worcester State Hospital suffering from dementia paralytica   The treatment consists of insulating the recumbent patient with wrappings and passing high frequency alternating currents through his body   In the course of 2 or 3 hours the patient's temperature may be elevated to 106 ± °F   After turning off the current, several hours are usually required for the temperature to return to normal   Clinical records of pulse and respiration have been kept on some 70 subjects   Each subject received approximately ten treatments   The observations of pulse and respiration as a function of temperature were taken every 10 or 15 minutes throughout the course of a typical 4 hour treatment

Fig 1 is a modified reproduction of a typical clinical chart showing the course of pulse frequency and respiration as a function of the rectal body temperature measured with a calibrated resistance thermometer   Three experiments with the same patient, performed on different days, are shown in this figure   The scale of respiratory frequencies is small and the precision of reading is rather low   The pulse frequency scale is larger and the data are more reliable than are those for respiration   The records of respiration, pulse, and temperature, in general, are fairly symmetrical for rising and falling temperatures   In Arrhenius equation plots, to be discussed presently, no consistent differences were found for the rising and falling values   The black rectangles of Fig 1 indicate the duration of application of the current which elevates the patient's temperature

Fig 2 is a composite plot of data obtained from ten treatments of
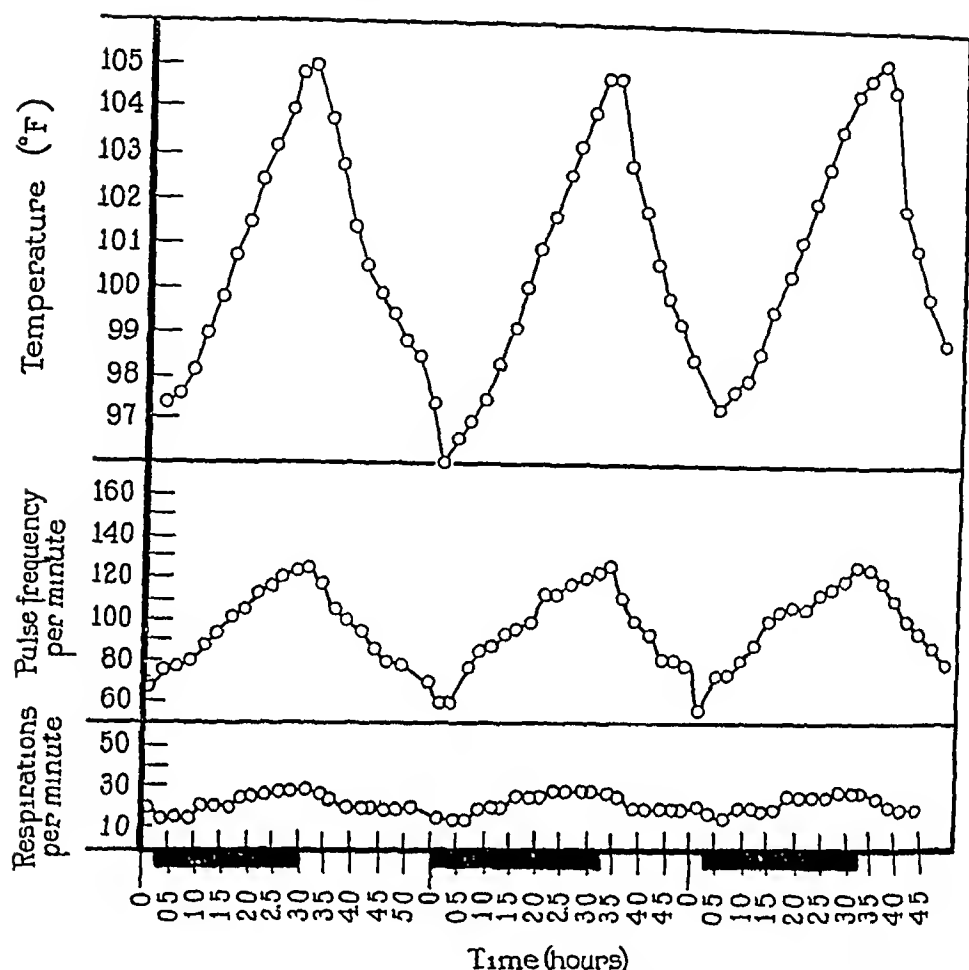
FIG 1 Modified reproduction of a typical clinical data sheet showing three experiments on one individual, each of about 5 hours duration. Pulse and respiratory frequencies are recorded as a function of the internal body temperature. The solid rectangles indicate the duration of the passage of the alternating current used to elevate the body temperature

the same patient administered on different days. The data are plotted according to the Arrhenius equation

$$\ln \frac{k_1}{k_2} = \mu/2 \left(\frac{1}{T_2} - \frac{1}{T_1} \right)$$

where k_1 and k_2 are the respective pulse frequencies at temperatures T_1 and T_2 and μ is the temperature characteristic. The variation of distribution of points of Fig 1 is large, but if it be assumed that the

Arrhenius equation holds and a straight line be drawn through the points, a value of $\mu =$ about 29,000 calories is obtained

As in investigations of the effect of temperature on physiological processes in poikilothermic animals, variation of the data is found to be less for single experiments than for averages of many experiments   This is due in part to fluctuations in the position of the curve as determined by its intercept on the ordinate axis, quite aside
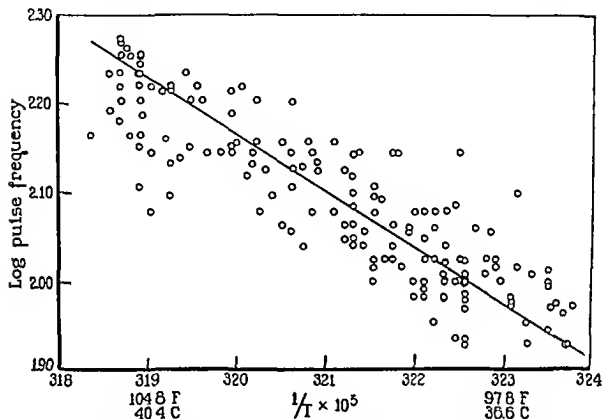
FIG 2 Arrhenius equation plot of heart beat frequency (pulse) as a function of temperature, yielding a temperature characteristic of 29 000 calories   164 observations from ten experiments on one individual are plotted   The value of μ is the arithmetic mean obtained by determining the temperature characteristics of each of the ten experiments separately

from fluctuations in the slope of the line, which determines the magnitude of μ

The temperature characteristic of 29,000 calories was obtained by plotting the data of each of the ten experiments separately, and averaging the value of μ for each experiment   As may be seen in Figs 3 and 5 the scatter of data for one experiment is not excessive   Fig 3 shows the results of two experiments on two individuals in

which pulse *vs* temperature is plotted according to the Arrhenius equation   The ordinate scale does not correspond to the absolute frequencies   The slopes of the lines are seen to be the same (μ = 29,500)
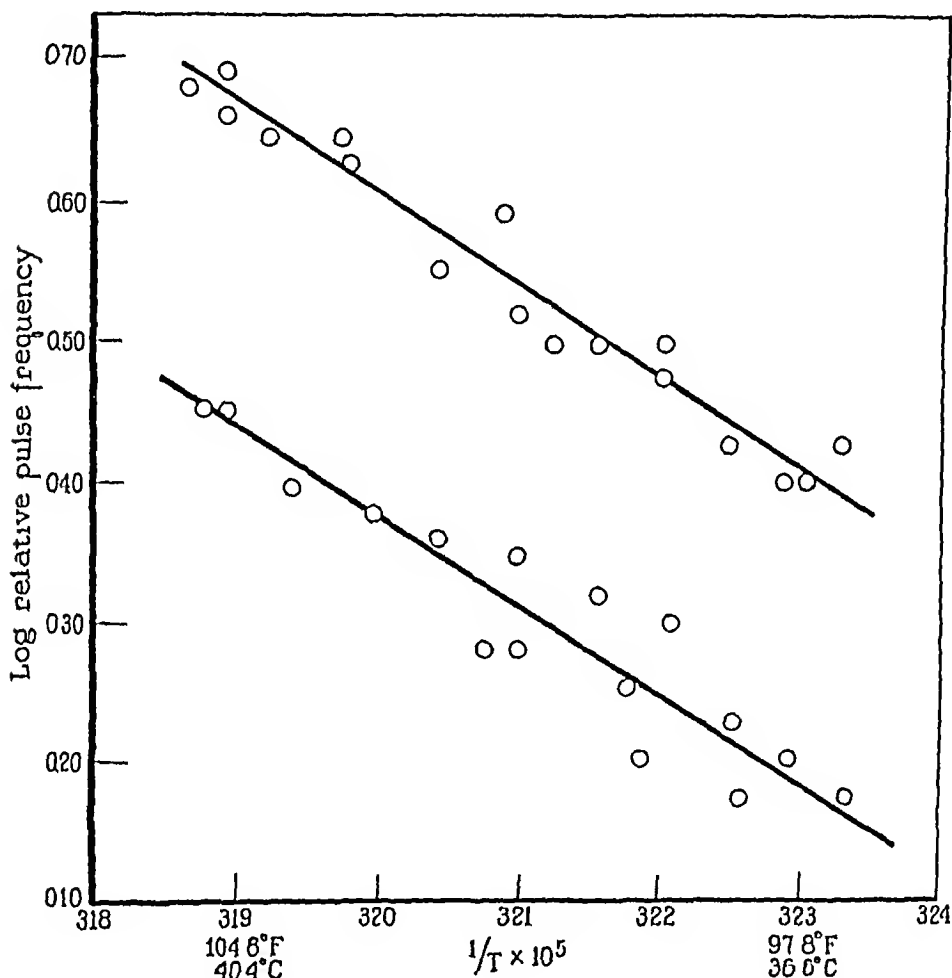
Fig 3 Typical Arrhenius equation plots for two experiments on different individuals

The considerable spread of data in Fig 2 in a large measure results from the fact that, despite considerable constancy in the value of μ (*cf* Table I), differences in pulse rate were found to occur in the same individual on different days, thus resulting in somewhat different intercepts of the line with the ordinate axis from day to day   Von

Korosy, cited by Loeb (1916), found variations among resting individuals in pulse frequency of from 42 to 108 beats per minute The constancy of μ values illustrated in Table I is, therefore, especially interesting, suggesting a high degree of chemical specificity in the pace making reaction despite differences in the absolute heart rates of individuals, and to a lesser degree in day-to-day fluctuations of the pulse for the same individual

TABLE I

Temperature Characteristics for Human Hearts

Patient	No of observations	Experimental day	μ
A	124	5 day mean	28 100
B	202	10 day mean	30 300
	18	2nd day	29,500
	18	6th day	29,300
C	183	10 day mean	29 900
	18	3rd day	30 000
	18	4th day	28 800
	18	10th day	30 400
D	223	10 day mean	29 900
E	164	10 day mean	29 000
Mean			29 400

Table I shows temperature characteristics obtained from five patients selected at random from the group of seventy The mean μ for 45 experiments involving 896 observations is 29,400 calories

The value of the temperature characteristic agrees well with one of the smaller modal groups ($\mu = 29,000$) obtained by Crozier (1925–26) from the analysis of many determinations of this constant for different physiological processes in poikilothermic organisms

Fig 4 shows the data of one typical experiment relating frequency of respiratory movements to temperature The value of μ from sixteen observations is roughly 41,000 calories The respiratory data show, in general, a greater scatter than does the heart frequency data,

and the data are less reliable   The values of μ obtained for respiratory movements are also much more variable, as is indicated by comparing Tables I and II
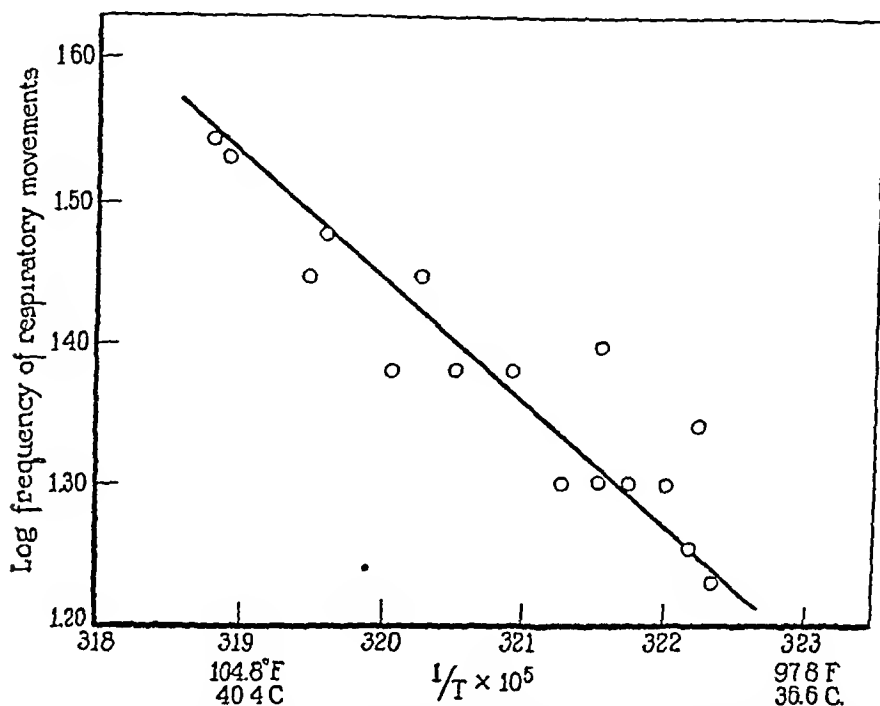
FIG 4 Typical Arrhenius equation plot of respiratory movements as a function of temperature, yielding a temperature characteristic of 41,000 calories

TABLE II

Temperature Characteristics for Frequencies of Respiratory Movements of Five Patients Selected at Random

Patient	No of observations	μ
B	17	44,300
F	17	34,700
G	16	41,200
H	22	38,400
I	88	43,800

From Fig 4 and Table II it is clear that not much may be said concerning the value of μ for respiratory movements, other than that it is consistently greater than that for the heart rate   Values of μ of

the order of 40,000 calories are seldom encountered in poikilothermic organisms During a treatment a patient may lose as much as 5 to 6 pounds of water through the skin and respiratory passages The lack of precision of the respiratory data makes it impossible to be certain that the Arrhenius equation fits, although no systematic failure of the equation was detectable The marked accelerations of respiration with increasing temperatures are probably the resultant of the control of the respiratory center by homeostatic neurological mechanisms involved in temperature regulation by elimination of water This effect probably masks the normal pace making mechanism of the respiratory center It is, therefore, doubtful if the value of $\mu = 40,000 \pm$ calories is at all comparable to values of temperature characteristics obtained from respiratory movements of poikilothermic animals

<center>II</center>

Recent studies of electrical potentials from the central nervous system have indicated that units in the central nervous system may be spontaneously active discharging impulses over efferent fibers at frequencies directly proportional to the rates of chemical changes going on in the centers Spontaneous fluctuations of potentials have been recorded by Adrian and Buytendijk (1931) from the isolated brain stem of the goldfish at frequencies corresponding to normal opercular breathing rhythms Similar spontaneous discharges from the respiratory center have been found from isolated ganglia of the beetle, *Dytiscus marginalis* (Adrian, 1931) Spontaneous activity in the central nervous system, not involving the respiratory center, has been recorded by other workers (Fischer, 1932, Bishop and Bartley, 1933a, 1933b, Bartley, 1933, Gerard, Marshall, and Saul, 1934, Prosser, 1934)

Investigations of the effect of temperature on the frequency of rhythms of activity of central nervous origin in poikilothermic animals have been made by Crozier and others (*cf* especially Crozier, 1924–25) Such diverse phenomena as the frequency of chirping of crickets, the frequency of flashing of fireflies, the speed of creeping of insects, follow the Arrhenius equation and yield specific temperature characteristics These studies have antedated observations of spon

taneous central nervous activity obtained by the electrical recording methods and are consistent with the notion of continuous chemical mechanisms in the central nervous system releasing motor discharges at frequencies directly proportional to the velocities of the mechanisms   It is interesting that Crozier and Stier (1924–25), for example, found a critical increment of $\mu = 16,500$ calories for the frequency of opercular breathing of goldfish before Adrian and Buytendijk recorded the spontaneous electrical effect from the brain stem

These matters, together with the finding that the "spontaneous" frequency of the repetitive discharge of nerve impulses from the peripherally unstimulated neuromasts of fish obeys the Arrhenius equation, led one of us (Hoagland, 1933) to consider that the estimation of durations of time by man might depend upon the rate of continuous underlying chemical events in the nervous system   If this were true, the frequency of counting seconds should follow the Arrhenius equation and might yield a significant critical increment   Naive subjects whose body temperature was altered by diathermy, as well as those whose temperature varied as a result of fever, were asked to count to 60 at a rate of what they believed to be 1 per second   The counting was found to be faster the higher the temperature, in accordance with the Arrhenius equation, yielding a μ of 24,000 calories   The fact that the value of μ coincides with one of the modal peaks found for temperature characteristics of poikilothermic animals (Crozier, 1925–26) suggests that the pace-making reaction, upon which judgments of duration are based, may be of the nature of an irreversible chemical reaction and may be catalyzed in a specific way corresponding to $\mu = 24,000$ calories—a value which has been found associated with certain cellular oxidation processes

The lower curve of Fig 5 shows a plot of the effect of temperature on judgments of duration for six subjects (cf Hoagland, 1933)   On the same figure are typical plots showing the effect of temperature on the pulse frequency for two other subjects   The plot was made by eliminating absolute differences in the speed of counting, by telescoping the ordinates so as to bring the data for the estimation of duration for all of the subjects together on the graph, thus showing the uniformity of the slope of the line which determines the value of μ

A similar procedure was used for eliminating absolute differences in heart rates of the two patients at the same temperatures

The values of μ for the pulse and for the speed of counting are distinctly different Whatever may be the chemical pace maker mechanism for judgments of short durations, it is clearly not the
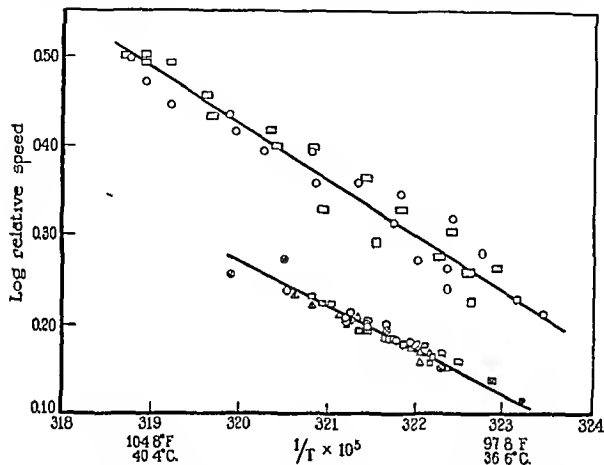
FIG 5 Arrhenius equation plots of pulse data from two patients (upper curve) and for the effect of temperature on estimations of duration (lower curve), six subjects

The positions of the sets of data with respect to the ordinate axis are arbitrary The curves are arranged to show the differences in the slopes of the lines for the two functions

same as the chemical determinant for the heart beat The suggestion, often made, that the pulse may serve as a sort of master clock deter mining one's sense of duration is evidently not tenable

SUMMARY

The value of $\mu = 29,400$ has been found for the human heart beat over the temperature range of approximately 4 7°C This value is

different from that of 24,000 calories which has been obtained for the effect of temperature on judgments of short durations

The evidence indicates that the estimation of short time intervals is controlled by a chemical master reaction which is independent of the pulse rhythm

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PROTOPLASMIC POTENTIALS IN HALICYSTIS

IV VACUOLAR PERFUSION WITH ARTIFICIAL SAP AND SEA WATER

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Bioelectric and various other effects are usually studied under the influence of changes in the solutions applied to the outside of living cells It is needless to emphasize the interest of similar alterations in internal solutions, but the difficulty of producing these has limited their experimental use A few such changes have been made by injection into vacuoles, both in large cells such as *Valonia*,[1][2] and in various small cells by microtechnique[3] But simple injection has several disadvantages Because of the limited extensibility of the cell wall, or because of other reasons, it is often possible to inject only small amounts of solution, sometimes only after previous extraction of some sap[1] or by allowing some to escape around the point of injection,[2] both of which are undesirable for bioelectric studies Not only is it difficult to determine the initial concentration of the injected substance in the sap, but this may soon change by diffusing away, reacting with cell constituents, or by precipitation Values of pH or of oxidation reduction potential experimentally produced by injection are particularly liable to such alteration through the metabolic activities of the cell It is also usually impossible to remove a given substance from the sap (save by some precipitation reaction) or to

[1] Blinks L R , *J Gen Physiol* , 1928–29 **12**, 207

[2] Jacques A G and Osterhout W J V *J Gen Physiol* 1928–29, **12**, 209

[3] Chambers, R in Cowdry E V General cytology Chicago University of Chicago Press 1924, 235   Chambers R , and Reznikoff P *J Gen Physiol* , 1925–29, **8**, 369   Chambers R in Cold Spring Harbor symposia on quantitative biology , Cold Spring Harbor Long Island Biological Association 1933 **1**, 205 Cohen B in Cold Spring Harbor symposia on quantitative biology , Cold Spring Harbor Long Island Biological Association 1933, **1**, 214

produce very much dilution of the sap as a whole   Finally, only one injection can usually be made in a given cell, so that it is impossible to investigate a series of effects, or to return later to normal conditions to test the state of the cell

It is therefore not surprising that most of the injections have been small amounts of various indicators,[3] designed to show existing conditions in the sap rather than to alter it, or small amounts of toxic materials (e g , manganese[2]) or very active salts (such as calcium and magnesium[3]), to compare internal and external effects

It is obvious that if a second, escape, tube could be inserted as well as the injection tube, and both kept in place without injury, many more changes could be accomplished   By a continuous flow the natural sap could eventually be replaced with new solutions, containing either deficiencies or additions of any given constituent, in known concentration, and constantly renewed to reduce metabolic changes   Various treatments could be applied in succession, and natural or artificial sap restored at any time to test reversibility and recovery

Such a perfusion method has been applied with success to the large multinucleate cells of *Halicystis*, and is described in the present paper While developed primarily to duplicate the pH effects produced by the penetration of ammonia, as described in Paper III[4] of this series, the substitution of various solutions such as artificial sap and sea water for natural sap in the vacuole, has further emphasized the essentially independent, inherent nature, of the protoplasmic potential in *Halicystis*, and is here presented from that point of view   Other uses will be evident

Method

The method is a modification of an unpublished one developed by Mr L B Damon, to provide for flow of sap through the vacuoles of *Valonia* cells, with simultaneous electrical contact through one or both of the perfusion tubes   In Damon's device, two glass capillaries were inserted either at separate points on the cell, or concentrically, a smaller inside a larger   While the *Valonia* cells lived fairly well when so impaled, considerable difficulty was met in their perfusion, owing to the presence in the sap of small masses of transparent jelly, which clogged the exit tube and were very difficult to remove

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[4] Blinks, L R , *J Gen Physiol* , 1933–34, **17**, 109

Fortunately the cell sap of *Halicystis* does not contain such jelly, the remains of the motile gametes sometimes present being small enough to pass readily through the capillaries, or to be removed by reversing the flow, or cleaning the capillaries with fine wires On the other hand owing to the different mechanical properties of the *Halicystis* cell wall, it was found practically impossible to insert either the two separate or the large concentric capillaries of Damon's scheme, without
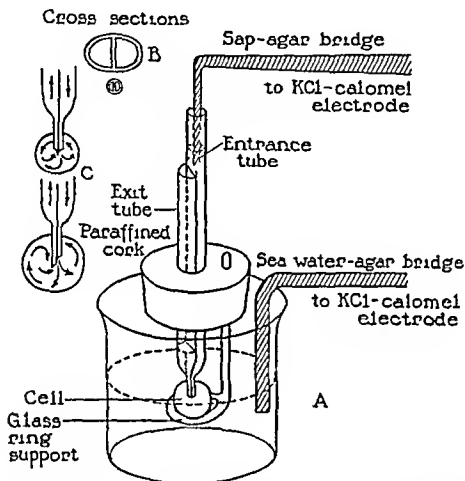
FIG 1 *A* apparatus for perfusing cells of *Halicystis* (incidental clamps and supports are omitted) *B* cross sections of double tubes and capillaries *C* tips of capillaries opening at angles to each other or at different levels, with circulation of fluids in the vacuole (as shown by added dyes)

collapse of the cell. Fused parallel capillaries were therefore substituted, making only one puncture sufficiently small to prevent collapse

Two ordinary glass tubes were first employed fused at their tips or along part of their length But the median wall remained of double thickness and was diffi cult to fuse and anneal making for separation or cracking Special double tubing was then constructed of the cross-section shown in Fig 1, *B* This is made by opening out one tube longitudinally and fusing its edges to another tube flat

tened along one side   It was convenient to have the latter project somewhat, in order to attach rubber tubing leading to a reservoir   The capillaries were easily drawn on the double tube, the median wall remaining intact, and separating the flow channels to the very tip   By pulling out the tips several times in a very fine flame it was possible to make the walls very thin while the orifices remained large enough to admit a fine wire for cleaning   The total outside diameter was usually not over 1 mm

The tips were cut or broken off under a magnifying glass so that the orifices occurred at slightly different levels, or at angles to each other, this made for more efficient stirring in the vacuole, and less immediate mixing of the entering and leaving streams   Examples of the tips are shown in Fig 1, C

The cells were firmly supported from below on glass rings, the capillaries being inserted from above by sliding down through the paraffined cork   This arrangement made a compact, rigid unit, which could be kept in a wide mouthed bottle until needed, and transferred to the measuring apparatus with a minimum of shaking or torsion of the protoplasmic seal around the capillary

The capillaries (usually sterilized before using) were inserted empty, whereupon enough sap gushed from the impaled cells to fill the tubes for a few millimeters above the tips   This sap maintained a slight head while the wound was healing, the cells being kept for this time very quietly just below the surface of sea water in bottles   Recovery often occurred in 30 to 60 minutes, but better results were obtained if the cells were left overnight before perfusing   The sap usually rose somewhat higher in the tubes during this time, in a manner corresponding to the recovery of turgidity after puncture, as previously described

The P D across the protoplasm reached during this time the high positive values characteristic of the species,[5][1 c], about 68 mv in H $Osterhoutii$, and about 80 mv in H $ovalis$ When appreciably lower values persisted, an imperfect seal was usually evident around the capillary, lifting the cells part way out of the sea water then caused most of the normal P D to appear[6]   This procedure was also useful when it was suspected that perfusion was inadequate in the upper part of the vacuole   The capacity of relatively small uninjured areas of $Halicystis$ protoplasm to give large P D 's even when short circuited by fairly large injured areas, is quite remarkable, though in keeping with the production of appreciable currents for long periods

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[5] Blinks, L R , J Gen $Physiol$, 1932–33, **16**, 147

[c] In this respect these cells agreed with the freshly impaled ones of $Valonia$ (Osterhout, W J V , Damon, E B , and Jacques, A G , J Gen $Physiol$, 1927–28, **11**, 193) which showed low P D 's when completely immersed, but nearly normal ones when only the lower tip was immersed in sea water   The situation also recalls that involving a new solution on the surface of $Valonia$, where the values are too low if only the tip touches the new solution, the rest being still imbibed with sea water (Damon, E B , J Gen $Physiol$, 1929–30, **13**, 207)

Electrical connection to calomel electrodes was made by agar bridges as shown in Fig 1, *A* These were made up with artificial sap or with sea water, to avoid contamination of the perfusing fluids by KCl (although this is shown later to have little or no effect) The KCl junction was made at their farther ends Potential measurement was by compensation, with a galvanometer as null instrument. Small current drains during balance had no effect on the P D, which stays up even under 5 to 10 microamperes flow, produced when the cells are short circuited through the galvanometer and the resistance of the capillaries (usually 10 000 to 20 000 ohms) The P.D has the sign of the outside solution *s e*, is positive when the positive current tends to flow outward across the protoplasm

Perfusion was readily accomplished by raising the level of fluid in one of the tubes (usually the longer one) As the fluid emerged in the exit tube it was removed continually by slight suction through a drain tube (not shown in Fig 1), or from time to time with a long narrow pipette Similarly the fluid in the entrance tube was renewed either from a reservoir, or when only small amounts were available (as with natural sap) it was recirculated by frequent pipetting from the exit tube The speed of perfusion depends of course upon the hydrostatic pressure and the diameter and length of the capillaries For especially fine ones it was necessary to attach a thistle tube to the entrance tube for greater head but this was usually unnecessary larger capillaries being less likely to clog or to emit a rapid stream (which might wash away the protoplasm opposite the opening) 8 or 10 cm head was usually sufficient to produce a good steady flow, *e g* 1 cc in 5 minutes and since the volume of the cells was seldom over 0 5 to 1 0 cc., this gave fairly rapid replacement of the vacuolar fluids

The extent of replacement and efficiency of stirring in the vacuole, were of course highly important, they could be determined by adding a tracer to the entering solution (*e g* a small quantity of dye such as methylene blue or cresyl blue) The depth of color in the entrance and exit tubes could then be directly compared as in a colorimeter separated only by the median wall, the stirring in the vacuole could also be seen directly through the green protoplasm Typical perfusion streams are shown in Fig 1 Stirring and mixing were of course better, the faster the entering stream but this could not be pushed too high from danger of injuring the protoplasm Small masses of gametes left in the vacuole from previous reproductive periods were also good indicators of stirring and were often seen to be in violent motion to be eventually broken up and lost through the exit tube Stratification of the tracer dye was sometimes evident when perfusion was very slow showing the new solution to fill only the lower half, or even a hand in the middle, of the cell Such stratification depends of course upon the specific gravities involved and was more frequent when solutions more dense than the sap were perfused It was thus more troublesome in *H Osterhoutii*, with sap of low density [7] (causing the cells to float) than in *H ovalis* with sap about as dense

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[7] Blinks, L R and Jacques A G, *J Gen Physiol* 1929–30 13, 733

as sea water,[8] and was of course worst when glucose, sucrose, or other "indifferent" substances were added to increase osmotic pressure   In these cases, most of the vacuole above the level of the entering orifice remained uncolored by the tracer, or became colored only very slowly   This unstirred region could be reduced by inserting the capillaries only a short distance into the vacuole, by gently tipping and revolving the cells about 45° from the vertical, or by inserting the capillaries from below, so that a rising entrance stream would aid the mixing

When densities were adjusted, good mixing was usually evident throughout the vacuole in about 5 minutes, especially around the vacuolar surface of the protoplasm, (eddies or dead spaces tending to be toward the center, as diagrammed in Fig 1, C)   This hastens the protoplasmic effects, although there probably remains a very thin unstirred layer immediately next to the protoplasm, across which diffusion still remains the governing factor   That this is fairly thin, and diffusion rapid, is shown by the speed of certain effects, such as those following pH changes, or deficiencies of essential salts such as potassium and calcium, mentioned below Theoretically of course, perfect mixing through the entire vacuole, and complete substitution of new solutions for the cell sap, would take an infinite time, practically, however, the solution in the exit tubes soon becomes identical (by color of dye, etc) with the entering solution, and the bioelectric effects occur, if at all, in 5 or 10 minutes with a reasonable speed of perfusion

The speed and characteristics of circulation having been established in typical dead and living cells, it was not necessary to add tracer dyes for most experiments, their presence, however, had no perceptible effect, and they were used when any question of adequate stirring arose   Other methods of testing the efficiency of perfusion were (1) adding an indicator either to the entering stream, or to the exit tube only, and comparing the pH of entering and leaving streams, (2) testing samples of the outflow for substances originally present in the sap but absent from the entering stream (*e g*, ammonia in *H Osterhoutii*), (3) raising and lowering the cells to different levels in the sea water, making evident the influence of unstirred regions upon the P D

The experiments have been performed at the room temperature of the laboratory, with yearly range from 15–25°C, and in ordinary north light   Very small fluctuations of either light or temperature occurred in a given experiment, and their effects were usually negligible   The effects of large variations of light and temperature will be discussed in other papers

H Osterhoutii was studied in Bermuda, *H ovalis* in California

Results of Perfusion
Natural Sap

It was of considerable interest to know whether the mere process of perfusion, with a flow of solutions on protoplasmic and capillary surfaces, changes of hydrostatic pressure, etc , had any influence upon

[8] Hollenberg, G J , *J Gen Physiol* , 1931–32, 15, 651

the observed P D across the protoplasm Such effects seem to be absent Not only is the P D practically identical when the hy drostatic pressure, and hence the turgor of the cell, is almost nil, or is raised to 50 cm of sea water, but also when the capillary is plugged with agar, or a stop-cock closed, so that the cell builds up practically normal turgor Furthermore, it is the same whether a good flow of sap is produced, or perfusion is stopped Streaming potentials, osmotic effects (as in a Donnan equilibrium), or "geoelectric" effects[9] are hence of little importance in the experiments, as would indeed be predicted from the high salt concentration (0 5 to 0 6 M) involved Conversely, no change of hydrostatic head was observed, even in a very fine capillary, when the P D was caused to reverse by the addition of ammonia, or when a fairly large electric current was passed across the protoplasm in either direction Anomalous osmosis, or electro endosmosis thus appears to be absent or insignificant

Similarly perfusion has no effect upon the P D due to exposure of the sap to air, or to foreign surfaces such as glass, rubber, etc Thus 2 or 3 cc of natural sap, extracted from several other cells, could be circulated continuously through the vacuole of an impaled cell, by pipetting repeatedly back from the exit to the entrance tube, for an hour or more, the P D remaining normal Even sap kept overnight could be so circulated, without effect on the normal P.D , unless pH or putrefactive changes had occurred

Artificial Sap

This established, it was of interest to know whether there were any peculiar properties of natural sap, such as small amounts of organic matter, which might be responsible for the high P D across the proto plasm This seemed unlikely, since natural sap applied externally does not reduce the P.D to zero, as would be expected from the re sultant symmetrical chain To test this further, various dilutions of natural sap, by admixtures of artificial sap or single salt solutions, were perfused It was found that these did not reduce the P D , as long as a reasonable degree of physiological balance was maintained (i e , Ca and Mg kept present about as in the natural sap, or at about

[9] Brauner L , *Jahrb wissensch Bot* , 1927, 66, 381

half the concentrations present in sea water)   In fact, it was found possible to perfuse *artificial sap* for long periods, to an extent that must have washed out most of the remaining natural sap, without reducing the P D   The addition of a small amount of glucose or sucrose kept the protoplasm from shrinking away from the wall during long perfusions with artificial sap, but was otherwise not essential to the procedure   It therefore seems unlikely that any organic constituent of the sap accounts for the P D   It might be imagined that the protoplasm was constantly secreting more of such matter directly into the unstirred layer of sap just within the vacuolar surface, but it seems reasonable that this would become considerably diluted during perfusion while the P D is not influenced

Sea Water

It remains to ask whether the inorganic constituents of the sap account for the P D   This also seems unlikely *a priori*, since in *H Osterhoutii* the sap differs only very slightly from the sea water[7] (chiefly by the absence of sulfate, and a much lower pH), furthermore in either species a large P D persists when artificial sap is applied externally [5,10]   But the inner surface might respond to certain substances quite differently from the outer, it was therefore essential to substitute sea water for natural or artificial sap in the vacuole   The cells were found to stand this treatment very well, especially if a small amount of sugar were added to prevent shrinkage of the protoplasm The results are complicated by the effect of pH (which with artificial and natural sap was maintained at 5 0)   If the sea water is brought to this value also, its effects when perfused in the vacuole are scarcely distinguishable from those of natural or artificial sap   This holds for both species of *Halicystis*   Thus in the absence of all known gradients except pH, i e with the salts of sea water present on both sides of the protoplasm, and constantly renewed by perfusion or stirring, a large P D (of 68 or 80 mv depending on the species) persists

If now, the pH gradient be also obliterated, there still remains a large P D   The outside sea water may thus be lowered to pH 5 by the addition of a trace of HCl   After a transient effect, large in *H*

10 Blinks, L R , *J Gen Physiol* , 1929–30, *13*, 223

Osterhoutii, very small in *H ovalis*, the P D largely or entirely recovers, remaining at 50 mv positive in the former, and still at about 80 mv positive in the latter species   This is in the absence of all known gradients, *i e* , with sea water of pH 5 on both sides of the protoplasm

Finally, normal sea water of pH 8 1 may be kept outside, and perfused as well through the vacuole   As reported earlier,[4] raising the pH of the vacuolar fluid in *H Osterhoutii*, either by penetration of ammonia, or by direct addition of NaOH, causes a profound change of the P D ,which reverses in sign, the critical pH of the sap apparently lying between pH 6 0 and 6 5   This occurs equally well whether natural sap, artificial sap, or sea water, is so raised in pH   Whatever the mechanism of this reversal, the significant point is that the P.D does not become zero, but may reach rather high values (20 to 40 mv negative) when the pH of the perfusing solution is raised to that of the outside solution   In other words with sea water of pH 8 1 inside and outside the protoplasm, a large (now negative) P.D is maintained

This situation in *H Osterhoutii* was expected to hold as well in *H ovalis*, since this species likewise undergoes a reversal of P.D in the presence of ammonia[4]   But this has not been found to be the case   Sea water of pH 8 1 (or even higher) may be perfused for long periods through the vacuoles of this species, with no reversal of P D , and with only a few millivolts decrease of the normal positive P D   Indeed there is sometimes no change of P D whatever   This unexpected indifference to the vacuolar pH is puzzling   It may be concerned with a higher critical pH for reversal (the ammonia threshhold is 3 to 10 times as high as in *H Osterhoutii*) or with a lower permeability of the protoplasm for hydrogen ions (suggested by the absence of pH effects externally as well)   Whatever the reason, however, this resistance to change offers perhaps the best evidence we yet have of the largely inherent nature of the P D in *Halicystis* *i e* , there is an 80 mv positive P D with sea water on both sides of the protoplasm

Effect of Potassium

Another interesting point comes out of the unchanged P.D when sea water is perfused in *H ovalis*   This species has about 0 3 M KCl

in the natural sap,[11] this is reduced to about 0 01 M when sea water is perfused, yet there is practically no reduction of P D   This is all the more remarkable since there is a very large effect[5] when KCl is increased on the external surface   The indifference of the vacuolar surface to such change nullifies the earlier suggestion made by the author that the higher P D in *H ovalis* might be due to the higher KCl content of its sap   Indeed, the converse experiment, earlier reported,[5] of raising the KCl content of *H Osterhoutii* sap by perfusion, has proved to be not very effective or lasting in raising the P D , and may have been confused with the pH effect, not then recognized [12]   The 10 or 12 mv difference of P D between the two species is thus apparently independent of the KCl gradient, and must be inherent, or "specific "

DISCUSSION

The chief result of the perfusion studies here described is increasing evidence that the P D across the protoplasm of *Halicystis* is almost entirely independent of any concentration gradients existing between the vacuole and exterior of the cell   This had been strongly indicated by the essential similarity of the cell sap of *H Osterhoutii*[7] to the

[11] Brooks, S C , *Proc Soc Exp Biol and Med* , 1929–30, 27, 209

[12] Similar indifference was met when filtered *Valonia* sap (containing about 0 5 M KCl) was perfused in the vacuoles of *H Osterhoutii*, the P D was not appreciably changed   It might also be added that in a few experiments where clogging did not interfere with perfusion of *Valonia* by the same method, a corresponding indifference of the vacuolar surface was found, when sea water was substituted for sap, the P D was practically unchanged   While too much faith is not placed in these *Valonia* perfusions (the cells did not live well afterward), it would appear that the vacuolar surface may have quite different properties from the outer surface, since potassium has a large effect when externally applied   This may have a bearing upon the suggestions offered to account for the shape of the KCl curves in *Valonia* (Damon, E B , *J Gen Physiol* , 1932–33, 16, 375)   There is, however, one striking potassium effect in *Halicystis* sap   A small concentration must apparently be present to maintain any P D whatever   Artificial sap, or artificial sea water, made up to contain no potassium (by cobaltinitrite test), quickly reduced the P D to zero, when perfused in *H Osterhoutii*, although without such effect when applied externally   Addition of a trace of KCl restored the P.D   This necessity of potassium in the balanced solution suggests the sensitivity of the heart to this element (there applied on the cell exteriors)

surrounding sea water, and further advanced by the large P D per
sisting in either species when natural sap or artificial sap was applied
externally [5] [10] There were, however, two possibilities remaining (1)
That certain organic constituents were absent from the artificial sap,
or were lost from the natural sap in the process of extraction, so that
they were lacking in the solutions applied externally, (2) That the
vacuolar surface of the protoplasm was alone sensitive to these sub
stances, the outer surface being indifferent

Change of solution in the vacuole itself was necessary to settle
these possibilities It is now seen that a large P D still persists when
artificial sap, containing no added organic constituents, or sea water,
exactly equivalent to the external solution, is perfused in the vacuole
While in one species the pH of the perfused solution influences the
sign of the P D it still remains large when the pH is the same on
both sides of the protoplasm

Some of these symmetrical chains may be reviewed

H Osterhoutis

Chain	P D	Sign of the outside solution (in the external circuit)
Natural sap | protoplasm | natural sap	40–50 mv	Positive
Artificial sap | protoplasm | artificial sap	40–50 mv	Positive
Sea water pH 5–6 | protoplasm | sea water pH 5–6	40–50 mv	Positive
Sea water pH 6 5–8 0 | protoplasm | sea water pH 6 5–8 0	20–40 mv	Negative

H ovalis

Chain	P.D.	Sign of the tside solution (in the external circuit)
Natural sap | protoplasm | natural sap	60–70 mv	Positive
Sea water | protoplasm | sea water pH 5–9 pH 5–9	80 mv	Positive

Obviously, such P D 's must depend upon some internal gradient
or asymmetry within the protoplasm itself Not only this, but the
constancy of the values with time and during appreciable current flow
demand an expenditure of energy which could eventually come only
from some metabolic activity, presumably an oxidation This should
be affected by temperature, light, oxygen supply, respiratory stimu
lants and inhibitors, oxidants, reductants, etc Some of these have

been studied and will be reported later   But it is obvious that many steps may lie between biological oxidations and manifest bioelectric potentials, one of these is the production of organic ions, which in diffusing outward, set up a potential difference   Such ions have been suggested by Osterhout[13] to account for radial asymmetries in *Valonia* and *Nitella*   If these were amphoteric, they might explain the reversal of P D in *Halicystis*

The nature of the gradients involved, the identification of the ions, if any, the source of energy, remain, of course, the essence of the problem, which merely becomes more clearly stated by the results of perfusion here reported   It is being studied further, and it is hoped that *Halicystis* may contribute to its solution, as well as to its statement

SUMMARY

Perfusion of the vacuole of living cells of *Halicystis* is described, the method employing two longitudinally fused capillaries as entrance and exit tubes   Natural sap, artificial sap, and sea water have been successfully perfused, with various additions and deficiencies, within the limits of physiological balance

In *H ovalis* the P D remains positive and scarcely reduced in value when normal sea water, at pH 8 1, is perfused in the vacuole   In *H Osterhoutii* the P D reverses in sign when the perfused solution has a higher pH than 6 5   In both cases a large P D persists when the solutions are the same on both sides of the protoplasm   In the absence of external gradients, there must be some internal gradient or asymmetry of the protoplasm itself to account for the P D   Since appreciable currents are produced, there must be some metabolic activity as a source of energy

The higher normal P D in *H ovalis* is not due to the higher KCl content of its sap (as earlier suggested by the author) since it persists nearly unchanged when sea water is substituted for sap

[13] Osterhout, W J V , *Bull Nat Research Council*, *No 69*, 1929, 170 (footnote 65), *Biol Rev* , 1931, 6, 382

THE SPREADING OF PEPSIN AND OF TRYPSIN*

By EVERT GORTER

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In this article the behavior of pepsin and trypsin in a monolayer under various conditions will be described. Both substances[1] show all the characteristic properties of other proteins. Pepsin is admirably suited for the study of the influence of positive ions, as its isoelectric point lies far to the acid side. On trypsin the influence of negative ions can easily be shown.

Pepsin[2]

The pepsin was obtained in the form of a 5 per cent solution in glycerol (1 cc. containing 50 mg of pepsin). It was diluted tenfold before use with 0 01 N hydrochloric acid (pH 2). 5 mm [3] were blown out of a calibrated micropipette on the surface of the water in a Langmuir tray according to the method which we have used for several years.

Effect of pH —The effect of pH on the amount of the spreading has been studied. For this purpose dilute hydrochloric acid solutions between pH 1 and 3, a 1/300 molar sodium acetate acetic acid solution between pH 3 6 and 5 6 (or 1/350 molar veronal acetate buffer solutions according to Michaelis), and mixtures of HCl 1/300 N and Na_2CO_3 1/300 N were used.

It was possible to show that, as a rule, the influence of pH is the same as that observed with other proteins. The maximum was again a

spreading of ± 1 sq m per mg. On the acid side, however, there exists a small minimum only, whereas on the alkaline side of the iso-electric point a pronounced minimum is observed (Fig 1)

It has been possible to demonstrate that the minimum (at pH 6 2) is strongly influenced by the addition of cations to the water in the tray. 1 milliequivalent of a bivalent cation has a distinct effect on the spreading: it tends to increase the size of the area at this point (Fig 2) It was impossible to find any difference between Mg^{++}, Ca^{++}, Sr^{++}, and Ba^{++}, all having the same effect in very small amounts ($\frac{1}{2}$ milli-
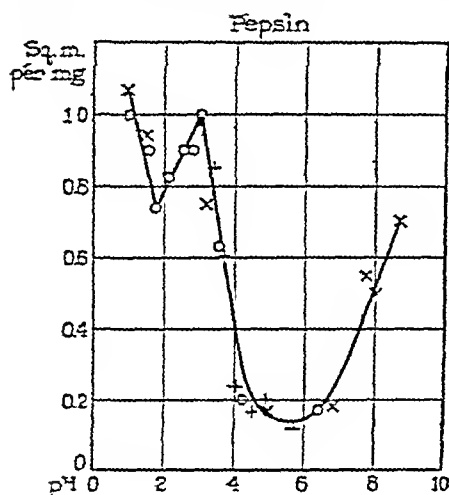
FIG 1
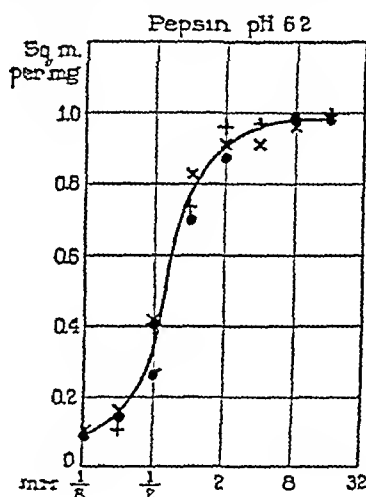
FIG 2

FIG 1 Influence of pH on the spreading of pepsin   The symbols $\circ$, $\times$, and $+$ indicate different samples of pepsin

FIG 2 Influence of bivalent cations on the spreading of pepsin   Ba is omitted, its curve is the same as the others given $\times$, Ca, $+$, Sr, $\bullet$, Mg

molar). This is in agreement with what we found on studying ovalbumin

Univalent cations have the same effect as have bivalent cations, but much larger amounts are necessary to produce an increase in the size of the area (Fig 3) Li^-, Na^+, and K^- behave differently according to their atomic number, the larger atom having the stronger effect (Fig 4)

The strong influence of polyvalent positive ions on the spreading at the alkaline side of the isoelectric point of a protein, like pepsin, can also be observed when making use of organic bases like spermine or
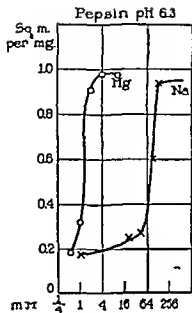
FIG 3
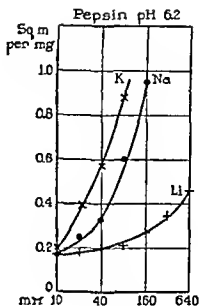
FIG 4

FIG 3 Influence of uni and bivalent ions on the spreading of pepsin ×, Na ○, Mg

FIG 4 Influence of lyotropic series on the spreading of pepsin ×, K, , Na +, Li
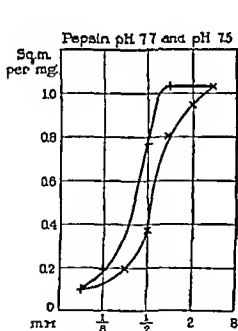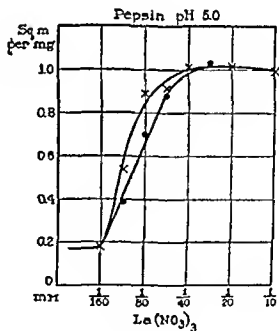
FIG 5

FIG 6

FIG 5 Influence of spermine and agmatine on the spreading of pepsin. + spermine ×, agmatine

FIG 6 Influence of time on the spreading of pepsin after addition of lanthanum nitrate   6 minutes, ×, 12 minutes

agmatine (Fig 5)   Exactly the same amount of these substances was sufficient to produce an increase in the spreading, when added to the water in the tray, at a pH of ±7 5 as was the case with Ca   A trivalent positive ion has a strong effect on the spreading at pH 5 0 $La(NO_3)_3$ has a distinct influence in a 1/8 millimolar solution   As in most of the experiments time has the same influence on the end result, chiefly when the spreading has an intermediate value (Fig 6)

It is obvious that pepsin is a protein that shows this influence extremely well, because its isoelectric point lies far on the acid side (pH 2 7)

With substances like guanidin, methylguanidin, creatin, and creatinine, *no effect on the spreading of pepsin was obtained at pH 6 2*, when the same amounts were used as in the case of monovalent ions like potassium and sodium

Trypsin

The trypsin was obtained in the form of a cake containing some ammonium sulfate and having a water content of ±70 per cent   It was dissolved in 0 001 molar hydrochloric acid   Solutions containing respectively 26 3, 27, and 28 mg cake per cc were used [3]

Quite a different type of curve was found, when plotting pH against size of spreading area (Fig 7)   A definite maximum of spreading at the isoelectric point could not be observed when using the original preparation containing ammonium sulfate

With trypsin having a minimum at pH 3 it was easy to study the effect of anions   It was possible to demonstrate

1   The valency effect in the series Cl^-, SO_4^{--}, and MTS^{---}* (Fig 8)
2   The lyotropic series Cl^-, Br^-, I^-, and CNS^- (Fig 9)

* MTS means methanetrisulfonic acid $CH(SO_3H)_3$

---

[3] The trypsin content of the cake was determined by means of the activity coefficient according to Northrop, modified somewhat by Dr Meyer   It was found that the cake contained 24 per cent active trypsin   This was assumed as the total protein content   Direct nitrogen determinations were not possible as the cake contained ammonium sulfate   This determination of the activity had the great advantage that it showed that the preparation had not been transformed into inactive material

Dr Northrop also sent trypsin in a dry state   It contained 92 per cent trypsin according to a N determination made by my collaborator Dr Meyer   This served for the last series of experiments
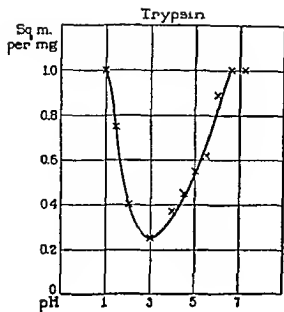
FIG 7
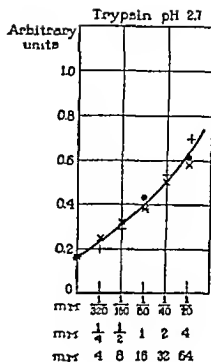
FIG 8

FIG 7 Influence of pH on the spreading of trypsin
FIG 8 Valency effect on trypsin   +, MTS, ×, SO₄, , Cl
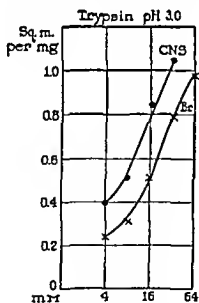
FIG 9
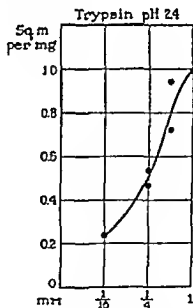
FIG 10

FIG 9 Lyotropic series   Influence on spreading of trypsin   Cl and I are
not given   Their curves are lying at the right place   , CNS ×, Br
FIG 10 Influence of glutathione on the spreading of trypsin.

The same observations could be made on the acid side of the iso-electric point when studying trypsin and when using substances like glutathione   This substance had a pronounced effect on the spreading when it was added to the water in the tray to make a $\frac{1}{4}$ millimolar solution, whereas the maximum was obtained with a $\frac{1}{2}$ millimolar solution (Fig 10)

On the other hand glutamic acid had no such influence and did not enhance the spreading, even when 528 mg per liter was used, that is, a 4 millimolar solution

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AN APPARATUS FOR PRESSURE MEASUREMENTS OF SPREADING SUBSTANCES*

BY EVERT GORTER AND WILHELM ADOLF SEEDER

(*From the Children's Clinic of the University Hospital, Leyden, Holland*)

Results of measurements of the spreading of proteins have been reported in the previous article The measurements were made with an apparatus differing in various ways from existing apparatus (Langmuir, Marcelin, and Adam)

It was essential in the construction of this apparatus to obtain an instrument by which it would be possible to make quick and accurate measurements [1] Also we tried to make handling of the instrument as easy as possible The latter feature is important for the use of the instrument in the clinical laboratory in, for instance, the estimation of spreading substances from the living organism such as proteins, lipoids, fats, and fatty acids

The method by which measurements are made is essentially identical with that by which Langmuir got his well known results

A free movable barrier C floats on the surface of the liquid in a shallow rectangular tray The barrier is connected by very thin platinum strips P_1P_2 (3μ thick) to the edge of the tray (Fig 1) A difference in tension of the surfaces at A and at B will cause a movement of the barrier C This movement is prevented by the fork of the torsion balance, and forces acting on the barrier can be transmitted to the balance and compensated by the torsion of a spring D The amount of torsion is read on the scale S Movements of the balance are detected by an optical system O and mirror M on the axis of the balance

[1] The apparatus is constructed by the mechanic G A de Vries at Leyden and can be supplied by C V Eiga Instrument Co Leyden Holland

For accurate measurements the barrier cannot be rigidly attached to the fork   Therefore the legs of the fork fit loosely in two slightly larger holes in the barrier C In this way only horizontal forces acting on the barrier will be transmitted to the torsion balance

A more detailed description of the instrument will now be given   The steel axis of the balance turns between two agates   These agates are normal watch agates with small circular holes in which the fine cylindrical points of the axis accurately fit   The stones are fitted in two cylindrical axes   One of the axes is fixed rigidly to the frame of the balance   The other one—by means of which the agate is accurately centered—is also mounted on the frame but in such a way that it can rotate around its axis   The index with nonius is fixed to
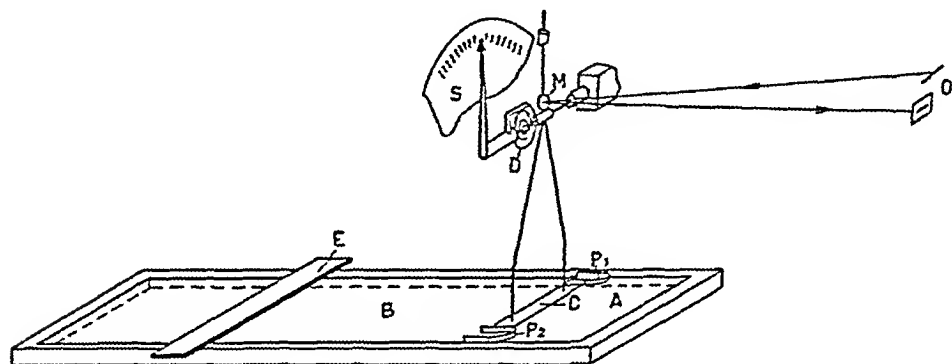
FIG 1

this movable axis, to which the free end of the spring is also attached   The steel axis fits exactly in the center hole of the spiral spring,—an alarm-clock escapement spring   For sake of linearity of torsion forces two springs are mounted on the axis with the spiral-winding in opposite directions   The scale on which the torsion of the springs is read can rotate around its axis, to obtain an easy zero adjustment

The next point to consider is the construction of the balance   Here it was tried to keep the balance system as light as possible without sacrificing rigidity   The vertical downward rods are tubes, obtained by drawing nickel-foil of 0 15 mm around an axis of 2 mm   To prevent chemical action of the fluid in the tray with the nickel, and capillary rise of that fluid in the narrow tubes, short capillary glass tubes with one end closed are cemented over the ends   The barrier is a thin mica strip (thickness 0 2 mm , other dimensions 0 8 x 12 cm)

At both ends are fixed two gilded brass holders for the platinum strips They are bent from hard brass foil in such a way that a small spring is formed to clamp the platinum strip (Fig 2)

The connection of the platinum strips with the sides of the tray is more complicated For easy cleaning of the surface of the fluid in the tray it should be possible to lift the balance from the water, the platinum strips also have to be lifted This is the reason why the strips are not fixed to the edges of the tray, but to a piece of gilded
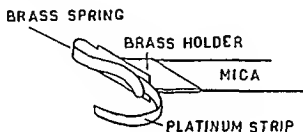
Fig 2
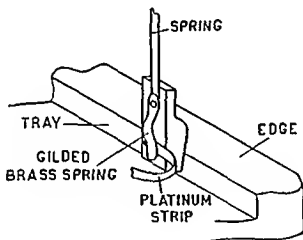
Fig 3

brass Here again a gilded brass spring is used to clamp the strip to the piece of brass This piece of brass is attached by a steel spring to the frame of the balance in such a way that the pieces of brass are pressed against the edge of the tray (Fig 3)

The frame of the balance can now slide up and down into two supports and can be lifted from the tray by a cog and pinion movement fitted to these supports This will be clear from the photograph of the apparatus The deviations of the balance are made visible by an optical system On the axis of the balance a small mirror is fixed

An optical system of the same construction as those in use for projection of galvanometer mirror movement is mounted to show movements of the axis of the balance   The sensitivity of this balance is 0 1 dyne/cm per scale degree   Tenths of degrees can be read

The next part to be described is the way in which the glass slide is moved over the tray   An arm in which the glass slides exactly fit can be moved by a long screw with a pitch of 1 cm   On a millimeter division fixed, parallel to the screw, on the support of the instrument the distance of the slide to the balance can be read (Fig 4)
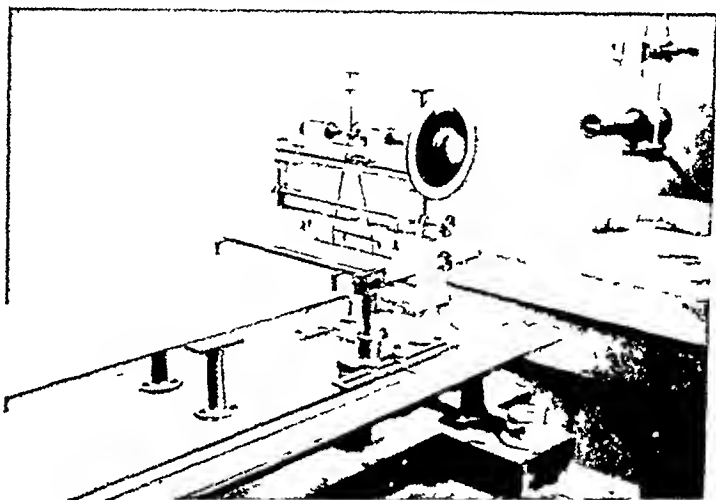
Fig 4

The tray is made of glass   At first metal trays and trays of cemented glass were tried   The trays obtained in the following way, however, give satisfaction   A mirror glass plate (17 x 63 cm) 10–12 mm thick is hollowed out over a rectangular surface (14 x 60 cm) by means of a sand-jet   The edges of this tray are slightly frosted   The use of glass has the advantage that it can easily be cleaned by a 1 per cent solution of hydrofluoric acid   All glass vessels used in our experiments are cleaned with this solution   The edges of the tray are paraffined by rubbing them with a piece of paraffin

After the edges have been covered with a sheet of paraffin, they are rubbed vigorously with a clean towel so that the paraffin sticks firmly to the glass   Then the paraffin is shaved off with a razor blade, leaving a very thin layer   Once more this layer is rubbed with a clean towel   The edges obtained in this way will last for about a week

Another method employed is the following   The tray is heated to about 80–100°C   By touching the edges with paraffin they are covered with a sheet of molten paraffin   This sheet is rubbed off with a clean towel   Again a very thin sheet of paraffin will remain on the edges, sufficient for spreading experiments

The glass slides are treated in the same way   Balance and platinum strips are treated with a very diluted solution of paraffin in ether

Salt solutions, etc , are obtained free from any spreading substances in the following way   The water used in the experiments is distilled in an all glass apparatus,—stops, corks, are all grease free   For the experiments with proteins, it is important to have water completely free from carbon dioxide   To destroy the organic substances, some chalk and potassium permanganate are added to the water in the distilling flask   This flask is automatically filled   By the bend loss of warm water to the intake regulator is prevented for the difference in specific weight of hot water and the cold water that enters the apparatus prevents convection at this bend   Also the chalk and potassium permanganate will stay in the distilling flask   Salts are recrystallized in grease free water—if possible they are heated to destroy organic material, and freed from contaminating substances, which lower surface tension

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CRYSTALLINE CHYMO TRYPSIN AND CHYMO TRYPSINOGEN

I Isolation, Crystallization, and General Properties of a New Proteolytic Enzyme and Its Precursor*

BY M KUNITZ AND JOHN H NORTHROP

(From the Laboratories of The Rockefeller Institute for Medical Research, Princeton, N J)

Kuhne (1) and Heidenhain (2) showed that extracts of fresh pancreas or freshly secreted pancreatic juice have no proteolytic activity The preparations become active when mixed with the enterokinase of the small intestine, as found by Schepowalnikow (3) or when the pancreas is allowed to stand in slightly acid solution The mechanism of this activation has been the subject of controversy for many years (4) Pavlov, Bayliss, Zunz, Wohlgemuth, Vernon, Delezenne, and others found the activation reaction to be catalytic and considered enterokinase to be an enzyme Hamburger and Hekma, Dastre and Stassano and Waldschmidt-Leitz found the reaction to be stoichiometric and considered that the enterokinase formed an addition compound with the inactive zymogen Vernon (5) found that activation could be caused by trypsin as well as by enterokinase but this was denied by Bayliss and Starling (6) The contradictory nature of the numerous experimental results indicates that there is more than one proteolytic enzyme in pancreatic extracts Vernon showed (7) that the activity, as determined by the clotting of milk, could be partially separated from the proteolytic activity, as determined by protein hydrolysis, and concluded that there were at least two enzymes He also showed that one of these was more stable than the other and that activation was caused by the less stable one

The crystalline trypsin previously reported by the writers (8) was obtained from pancreas which had been allowed to activate spontaneously The present experiments were carried out with fresh inactive pancreatic extracts in order to obtain the inactive form of the enzyme and to study the kinetics of activation

In the course of these attempts to isolate the inactive precursor of crystalline trypsin a crystalline inactive protein was isolated from inactive (cattle) pancreatic extracts   This protein was called chymo-trypsinogen   It cannot be activated by enterokinase but is changed into an active proteolytic enzyme by crystalline trypsin   The new proteolytic enzyme formed in this way was also crystallized and was called chymo-trypsin   It differs from chymo-trypsinogen in crystalline form, optical activity, and number of amino groups, and it is more soluble and less stable   The molecular weight and molecular radius are about the same as the corresponding values for chymo-trypsinogen

The new enzyme differs from the crystalline trypsin previously described in that it clots milk but does not clot blood and has a weaker action on protamines   It resembles crystalline trypsin in that it digests denatured proteins in slightly alkaline solution [1]

The results agree, in general, with Vernon's experiments since they show that there are at least two proteolytic enzymes present in activated pancreatic extract, trypsin and chymo-trypsin   Fresh, inactive pancreatic extracts contain at least two zymogens, chymo-trypsinogen and trypsinogen   Enterokinase transforms trypsinogen into trypsin and this in turn transforms chymo-trypsinogen into chymo-trypsin

Qualitatively this mechanism accounts for the peculiarly shaped curves frequently observed for the activation of crude pancreatic extract by enterokinase (5)   The activation of chymo-trypsinogen by trypsin is a simple catalytic monomolecular reaction but when this is superimposed upon the primary activation of trypsinogen by enterokinase the combined result yields a complicated asymmetrical curve   The transformation of chymo-trypsinogen into chymo-trypsin is accompanied by a change in optical activity and a slight increase in amino nitrogen   There is no detectable non-protein nitrogen fraction formed nor is there any significant change in molecular weight   The reaction, therefore, is probably an internal rearrangement, possibly due to the splitting of a ring   It is possible, however, that a small part of the molecule containing no nitrogen is split off although there is no evidence for this at present

[1] Waldschmidt-Leitz and Akabori (*Z   physiol Chem* , 1934, **228**, 224) have recently shown that pancreatic "proteinase" probably represents a mixture of trypsin and chymo-trypsin

Both the active and inactive form of the enzyme may be recrystallized repeatedly without change of properties   Denaturation or hydrolysis of the protein results in a corresponding loss in activity   There is reason, therefore, to believe that the preparations represent pure proteins and that the proteolytic activity is a property of the protein molecule

EXPERIMENTAL RESULTS

Isolation and Crystallization of Chymo Trypsinogen

The material used in these experiments was cattle pancreas removed from the animal immediately after slaughter and immersed in cold N/4 sulfuric acid   Acid prevents spontaneous activation and removes practically all the potentially active material from the pancreas while most of the inert protein is precipitated   The acid extract obtained in this way (when brought to pH 7 0–8 0) is rapidly activated by enterokinase but cannot be activated by small amounts of trypsin   A protein was crystallized from this extract which could be activated either by enterokinase or trypsin, it was called chymo trypsinogen   After repeated crystallization, however, the protein could not be activated by enterokinase but only by trypsin   The explanation of these apparently contradictory results is that the crude extract and the once crystallized protein contain trypsinogen and also some substance which inactivates small amounts of trypsin   When small amounts of trypsin are added to such preparations, therefore, no activation of chymo trypsinogen occurs since the trypsin added is inactivated, but when enterokinase is added sufficient active trypsin is formed from the trypsinogen to overcome the inhibiting action of the solution and so activate the chymo trypsinogen   The same result can be obtained by adding enough trypsin even in the presence of the inhibitor   The effect of repeated crystallization is simply to remove the last traces of these impurities, and the experiment is a good example of the efficiency of recrystallization as a method of purification

The mechanism outlined above was confirmed by mixing pure crystalline chymo trypsinogen with the mother liquor from the first crystallization and adding trypsin or enterokinase   The results of such an experiment are shown in Table I   As stated above, the recrystallized chymo trypsinogen is activated only by trypsin while the mother liquor from the first crystallization, or the chymo trypsinogen when

mixed with the mother liquor, cannot be activated by small amounts of trypsin but can be activated by enterokinase   The inhibiting ef-

TABLE I

Effect of Addition of Mother Liquor from Chymo-Trypsinogen Crystallization on the Activation of Chymo-Trypsinogen by Enterokinase and Trypsin

Quantity of material activated in 10 ml M/25 phosphate buffer pH 7 6	Chymo trypsinogen 0 4 mg protein nitrogen		Mother liquor 0 16 mg protein nitrogen		Chymo-trypsinogen 0 4 mg protein nitrogen + mother liquor 0 16 mg protein nitrogen	
Activating agent	Trypsin	Entero-kinase (20)	Trypsin	Entero-kin-ase	Trypsin	Entero-kin-ase
Quantity in 10 ml	0 002 mg protein nitrogen	2 ml	0 002 mg protein nitrogen	2 ml	0 002 mg protein nitrogen	2 ml
$[T\,U\,]_{ml}^{Hemoglobin}$ after 69 hrs at 5°C	0 002	<0 0001	<0 0001	0 0015	<0 0001	0 004

TABLE II

Inhibiting Effect of Mother Liquor on Digestion of Hemoglobin by Chymo-Trypsin and Trypsin

1 ml { Crystalline trypsin solution containing 0 03 mg protein nitrogen or Crystalline chymo-trypsin containing 0 07 mg protein nitrogen } added to 4 ml M/20

phosphate buffer pH 7 6 containing increasing amounts trypsinogen-mother liquor, activity of solution determined by hemoglobin method

Protein nitrogen in mother liquor, added, *mg*	0	0 04	0 08
$[T\,U\,]_{ml}^{Hb} \times 10^{-4}$ crystalline trypsin	7 2	6 3	5 6
$[T\,U\,]_{ml}^{Hb} \times 10^{-4}$ chymo-trypsin	8 8	8 8	8 8

fect of the mother liquor may be demonstrated directly by determining the effect upon the digestion of hemoglobin   The results of such an experiment are shown in Table II   The mother liquor inhibits

the digestion of hemoglobin by crystalline trypsin but not the digestion of hemoglobin by chymo trypsin

Isolation of Chymo Trypsinogen

The method finally adopted for the preparation of chymo trypsinogen is as follows

The pancreas is removed from cattle immediately after slaughter and immersed in cold $\frac{1}{4}$ normal sulfuric acid   Fat and connective tissue are removed and the pancreas minced in a meat grinder, suspended in 2 volumes of ice cold N/4 sulfuric acid, and the suspension allowed to stand in the cold room at 5°C overnight.   It is then strained through gauze on a large Büchner funnel and the precipitate resuspended in an equal volume of N/4 sulfuric acid and refiltered   The combined filtrates and washings are brought to 0 4 saturated ammonium sulfate by the addition of solid ammonium sulfate and the suspension filtered through soft fluted paper (S and S No 1450 $\frac{1}{2}$) in the cold room   The filtrate is brought to 0 7 saturated ammonium sulfate and the suspension allowed to settle in the cold room for 48 hours   The supernatant fluid is decanted and the suspension filtered with suction   The filter cake is dissolved in 3 volumes of water[2] and 2 volumes saturated ammonium sulfate added   The suspension is filtered and the precipitate discarded   The filtrate is brought to 0 7 saturated ammonium sulfate by the addition of solid ammonium sulfate or an equal volume of saturated ammonium sulfate   The suspension is filtered with suction   The filter cake is dissolved in 1 5 volumes water and brought to $\frac{1}{4}$ saturated ammonium sulfate by the addition of saturated ammonium sulfate solution   The solution is adjusted to pH 5 0 (brick red color with methyl red on test plate) by the addition of 5 N sodium hydroxide   About 1 5 ml per 100 ml of solution is required   The solution is allowed to stand for 2 days at room temperature (about 20°C)   A heavy crop of crystals gradually forms   They are filtered with suction   The isolation of the chymo trypsinogen is practically complete in one crystallization

[2] The volume of the semi-dry filter cake is usually determined by weight   The specific volume of the filter cake is assumed for convenience to be equal to one. The expression 'the filter cake is dissolved in n volumes of solvent   as used in the text, means that 1 gm of filter cake is dissolved in n ml of solvent

Recrystallization

The crystalline filter cake is suspended in 3 volumes of water and 5 N sulfuric acid added from a burette with stirring until the precipitate is dissolved   The solution is brought to $\frac{1}{4}$ saturated ammonium sulfate by the addition of 1 volume of saturated ammonium sulfate   An equivalent amount of 5 N sodium hydroxide is then added with stirring and the solution inoculated and allowed to stand at 20°C   Crystallization should be practically complete in an hour

If the crystals are to be used for the preparation of active chymotrypsin the crystallization should be repeated seven or eight times as otherwise difficulty is encountered in crystallizing the active enzyme

TABLE III

Fractional Crystallization of Chymo-Trypsinogen

Times crystallized	Optical activity in $\text{M}/10$ acetic acid, 25°C $[\alpha]^D_{\text{mg protein nitrogen}}$	Specific activity after activation, 18 hrs , 6°C in pH 8 0 м/50 phosphate buffer solution $+$ 0 0007 mg crystalline trypsin/ml		
		Hemoglobin $[T\ U]^{Hb}_{\text{mg protein nitrogen}}$	Gelatin viscosity $[T\ U]^{Gel\ V}_{\text{mg protein nitrogen}}$	Casein formol $[T\ U]^{Cas\ \Gamma}_{\text{mg protein nitrogen}}$
1	0 480	0 036	13 5	0 069
3	0 475	0 037	15 9	0 094
5	0 473	0 037	13 5	0 073
8	0 480	0 040		0 070
10	0 482	0 038	13 9	0 056

The above outline describes the preparation from fresh inactive pancreas   The preparations vary somewhat and occasionally the first crystals retain a brown coloring matter   This coloring matter may be removed by the addition of 2 volumes of saturated ammonium sulfate to the acid solution of the crystals   An amorphous precipitate is formed which carries down with it the coloring matter and which may be removed by filtration   Most of the enzyme may be recovered from the precipitate by washing the precipitate on the filter paper with N/100 sulfuric acid

The material may be isolated from frozen fresh inactive pancreas although this procedure is more troublesome   The gland must be frozen rapidly and immediately after removal   Ordinary commercial frozen pancreas is active and cannot be used   Crystals prepared from frozen pancreas frequently contain small amounts of foreign inert protein   This may be removed as follows   The crystals are dissolved in 3 volumes of water and sulfuric acid added, as described for recrystallization, and the solution neutralized by the addition of an equivalent
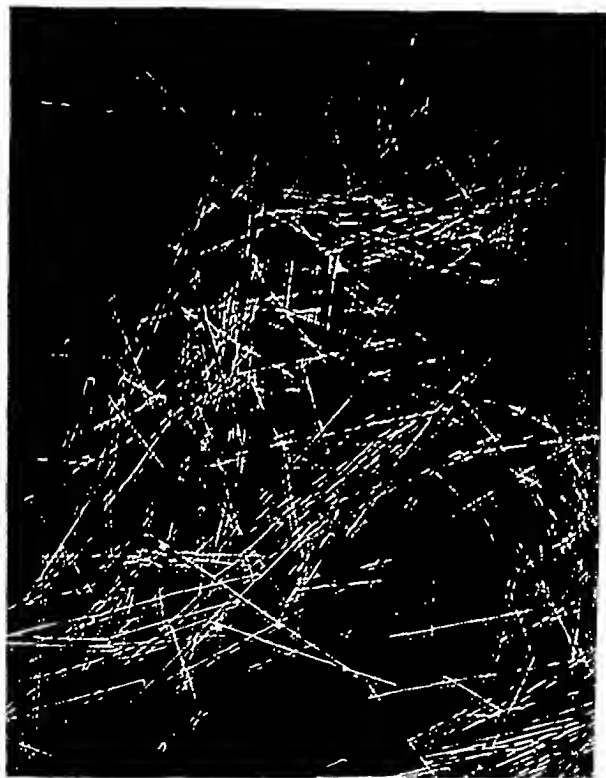
FIG 1 Chymo trypsinogen

amount of sodium hydroxide   A gelatinous precipitate appears and is filtered off The filtrate is acidified again with a few drops of 5 N sulfuric acid, 1 volume saturated ammonium sulfate added, and the solution brought to pH 5 0 with sodium hydroxide and inoculated

The preparation is conveniently carried out with about 10 fresh cattle pancreas   About 15 gm of once crystallized filter cake is usually obtained from 10 pancreas

The properties of the crystalline chymo-trypsinogen are constant through at least ten fractional recrystallizations as shown in Table III The crystals of chymo-trypsinogen are shown in Fig 1

Activation of Chymo-Trypsinogen

Chymo-trypsinogen after recrystallization has a variable and barely measurable activity equivalent to about 1/10,000 that of chymo-trypsin   This activity is probably due to the presence of traces of chymo-trypsin since the relative activity on various proteins agrees with that of chymo-trypsin

The chymo-trypsinogen could not be activated by enterokinase, calcium chloride, pepsin, inactivated trypsin, or by chymo-trypsin It could be activated by all commercial trypsin preparations tried and also by all crude active pancreatic extracts

Kinetics of Activation of Chymo-Trypsinogen by Crystalline Trypsin

Effect of pH —The effect of the pH of the solution on the rate of the activation of chymo-trypsinogen is shown in Fig 2   The curve resembles that for the effect of pH on the digestion of casein by trypsin and indicates that the reaction is related to the usual hydrolytic action of trypsin   However, as will be discussed below, no evidence for any actual cleavage of the chymo-trypsinogen molecule could be found

Effect of the Concentration of Trypsin —The activation follows the course of a monomolecular reaction and the rate is proportional to the concentration of trypsin added   This result is shown in Fig 3 (*cf* Table IV) in which the logarithm of the per cent of chymo-trypsinogen remaining at any time is plotted against the time   The resulting curves are all straight lines showing that the reaction is *monomolecu-lar*   The slopes of the curves are proportional to the concentration of

trypsin present showing that the rate of reaction is proportional to the trypsin concentration

Effect of the Chymo Trypsinogen Concentration —The per cent of the chymo trypsinogen activated at any time is constant and independent
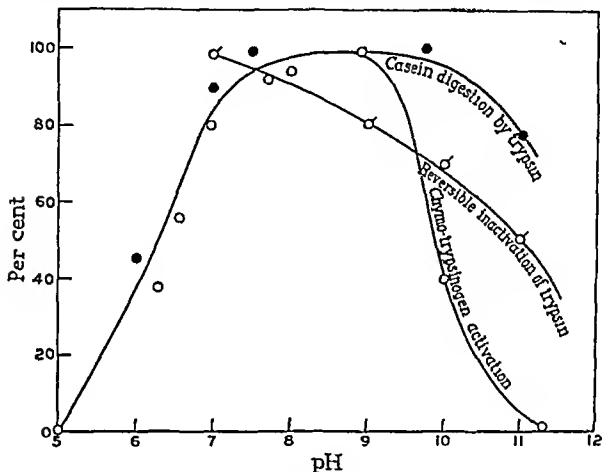
FIG 2 Effect of pH on rate of activation of chymo-trypsinogen (5 C) by trypsin compared with the effect of pH on rate of digestion of casein by trypsin (8) and the effect of pH on reversible inactivation of trypsin (10)

Activation mixture—5 ml chymo-trypsinogen solution in N/400 hydrochloric acid (1 mg protein nitrogen/ml) plus 1 ml trypsin solution (0 006 mg protein nitrogen/ml) plus 4 ml M/5 borate phosphate buffer of various pH Left at 5 C and activity determined by hemoglobin at intervals Rate of activation determined from these results

of the concentration of chymo trypsinogen This result is shown in Table IV The activation of chymo trypsinogen by trypsin, therefore, is expressed by the equation

$$-\frac{dG}{dt} = KTG$$

in which T = concentration of trypsin and G = concentration of chymo-trypsinogen, or on integration

$$KT = \frac{1}{t} \ln \frac{G_o}{G_t}$$

KT is the observed velocity constant (K') for any one concentration of trypsin   The value of K for unit trypsin concentration may be

FIG 3 Effect of trypsin concentration on rate of activation of chymo-trypsinogen (*cf* Table IV).

calculated and is found to be 670 per hour per mole trypsin/liter or 360 per hour per mg trypsin nitrogen/ml

In the preceding experiments the activity was determined by the rate of digestion of hemoglobin   The active enzyme clots milk and digests sturin so that the rate of activation was followed also by the

rennet action and by sturin digestion The per cent of activation, as determined by these three methods, is the same as shown in Table

TABLE IV

Activation of Chymo-Trypsinogen by Trypsin at 5°C

Effect of Concentration of Trypsin

Chymo-trypsinogen solution 0 26 mg protein nitrogen/ml. in 0 075 M phosphate buffer pH 7 6   4 ml. chymo-trypsinogen solution + 1 ml crystalline trypsin solution in M/400 hydrochloric acid at 5°C   1 ml samples taken into 4 ml M/70 hydrochloric acid   Activity determined by the hemoglobin method

$Log_{10} 100 \dfrac{\text{(Final activity} - \text{activity at time } t)}{\text{Final activity}}$ plotted against time in hours in

Fig 3

Effect of Concentration of Chymo-Trypsinogen

Solutions, etc., as above   0 002 mg crystalline trypsin/ml activation mixture

Concentration of chymo-trypsinogen mg protein nitrogen/ml..	0 42	0 105
Time	$[T\ U\ ]_{mg\ N}^{Ren.}$	
hrs		
0	0	0
0 5	3 50	3 46
1 0	6 20	5 04
2 0	7 00	7 06
7 0	9 00	8 60

TABLE V

Rate of Activation of Chymo Trypsinogen as Measured by Digestion of Hemoglobin, Clotting of Milk, or Digestion of Sturin

1 ml dialyzed chymo-trypsinogen solution containing 1 0 mg protein nitrogen/ml. + 1 ml crystalline trypsin solution (0 01 mg N/ml. $\approx$ 0 0016 $[T\ U\ ]^{Hb}$) + 3 ml M/10 pH 7 6 phosphate buffer 25 C   Sampled at intervals, 1 ml + 4 ml M/15 hydrochloric acid   Activity determined by various methods

Time at 25 C. *min*		15	30
Per cent final activity by	Hemoglobin method	77	100
	Clotting of milk.	84	100
	Sturin	78	100

V   This result indicates that these various substrates are all attacked by the same enzyme

in which T = concentration of trypsin and G = concentration of chymo-trypsinogen, or on integration

$$KT = \frac{1}{t} \ln \frac{G_o}{G_t}$$

KT is the observed velocity constant (K') for any one concentration of trypsin   The value of K for unit trypsin concentration may be

FIG 3 Effect of trypsin concentration on rate of activation of chymo-trypsinogen (cf Table IV)

calculated and is found to be 670 per hour per mole trypsin/liter or 360 per hour per mg trypsin nitrogen/ml

In the preceding experiments the activity was determined by the rate of digestion of hemoglobin   The active enzyme clots milk and digests sturin so that the rate of activation was followed also by the

rennet action and by sturin digestion   The per cent of activation,
as determined by these three methods, is the same as shown in Table

TABLE IV

Activation of Chymo Trypsinogen by Trypsin at 5 C
Effect of Concentration of Trypsin

Chymo trypsinogen solution   0 26 mg protein nitrogen/ml   in 0 075 M phos
phate buffer pH 7 6   4 ml chymo-trypsinogen solution + 1 ml. crystalline
trypsin solution in M/400 hydrochloric acid at 5 C   1 ml samples taken into
4 ml M/70 hydrochloric acid   Activity determined by the hemoglobin method
$Log_{10} 100 \frac{(Final\ activity - activity\ at\ time\ t)}{Final\ activity}$ plotted against time in hours in
Fig 3

Effect of Concentration of Chymo-Trypsinogen

Solutions etc   as above   0 002 mg crystalline trypsin/ml. activation mixture.

Concentration of chymo-trypsinogen mg protein nitrogen/ml...	0 42	0 105
Time	$[T\ U]^{Ren.}_{mg\ N}$	
hrs		
0 0	0	0
0 5	3 50	3 46
1 0	6 20	5 04
2 0	7 00	7 06
7 0	9 00	8 60

TABLE V

Rate of Activation of Chymo-Trypsinogen as Measured by Digestion of Hemoglobin,
Clotting of Milk or Digestion of Sturin

1 ml dialyzed chymo trypsinogen solution containing 1 0 mg protein nitrogen/
ml + 1 ml crystalline trypsin solution (0 01 mg N/ml $\simeq$ 0 0016 $[T\ U]^{Hb}$) +
3 ml. M/10 pH 7 6 phosphate buffer, 25 C   Sampled at intervals, 1 ml + 4 ml
M/15 hydrochloric acid   Activity determined by various methods

Time at 25 C. *min*		15	30
Per cent final activity by	Hemoglobin method	77	100
	Clotting of milk	84	100
	Sturin	78	100

V   This result indicates that these various substrates are all attacked
by the same enzyme

Change in Non-Protein Nitrogen during Activation —There is a slight increase in non-protein nitrogen during activation but the appearance of this non-protein nitrogen does not parallel the increase in activity (Fig 4)   It is probable, therefore, that the production of these non-protein compounds is a secondary reaction due to the gradual autolysis of the chymo-trypsin   The total amount of non-protein nitrogen found amounts to less than 10 per cent of the total nitrogen
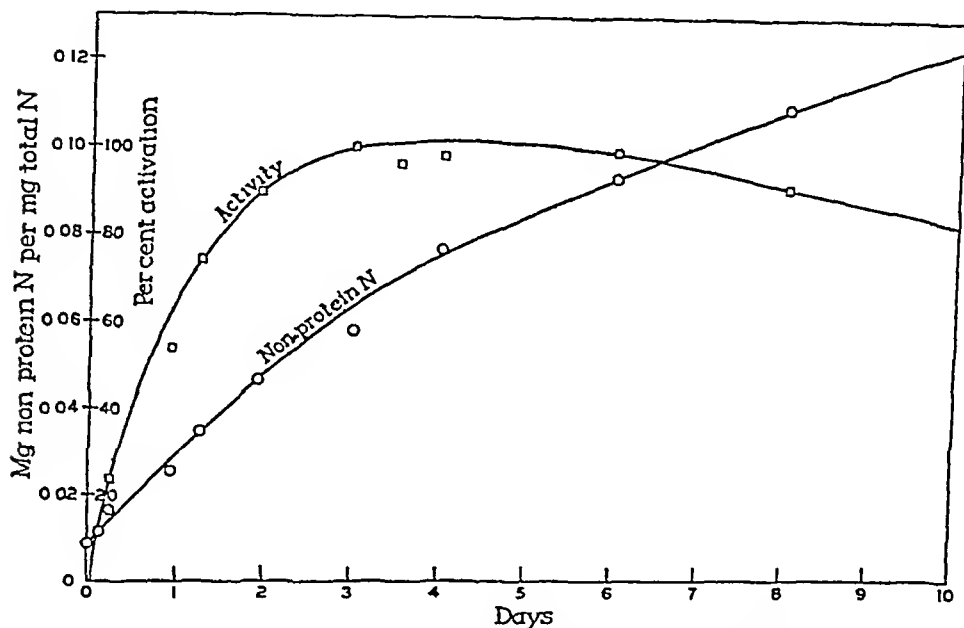
FIG 4   Increase in non-protein nitrogen during activation of chymo-trypsinogen by trypsin   10 ml chymo-trypsinogen solution containing 9 mg protein nitrogen/ml plus 1 ml M/1 pH 7 6 phosphate buffer plus 1 ml crystalline trypsin (0 0035 mg protein nitrogen) 5°C   Samples 1 ml plus 4 ml N/40 hydrochloric acid   Analyzed for non-protein nitrogen and hemoglobin activity

Spontaneous Activation

The activation experiments just described were all carried out in the presence of trypsin   There is, however, a very slow spontaneous activation   Less than 1 per cent of the chymo-trypsinogen is activated in a month at 5°C   There is no marked pH optimum but the reaction appears to go faster in weakly acid or alkaline solutions   It is probable, therefore, that it is an independent reaction and is not caused by minute amounts of trypsin

Isolation and Crystallization of Chymo-Trypsin

The final method adopted for the preparation of chymo trypsin from chymo-trypsinogen is as follows

The chymo trypsinogen should be recrystallized eight times 10 gm of crystalline chymo trypsinogen filter cake is suspended in 30 ml water and dissolved by the addition of a few drops of 5 N sulfuric acid 10 ml M/2 pH 7 6 phosphate buffer is added and a quantity of molar sodium hydroxide equivalent to the acid is also added About 0 5 mg crystalline trypsin is added and the solution left at about 5°C for 48 hours Any active trypsin preparation (of equivalent activity) may be used instead of the crystalline trypsin After 48 hours the solution is brought to pH 4 0 by the addition of about 5 ml N/1 sulfuric acid, 25 gm solid ammonium sulfate is added, and the precipitate filtered with suction

Crystallization

The filter cake is dissolved in 0 75 volumes N/100 sulfuric acid and filtered if the solution is not clear The clear filtrate is inoculated and allowed to stand at 20°C for 24 hours About 5 gm of crystal line filter cake should form

Recrystallization

The crystalline filter cake is dissolved in 1 5 volumes N/100 sulfuric acid, 1 volume of saturated ammonium sulfate is then added cau tiously until crystallization commences The solution is allowed to stand at room temperature and practically complete crystallization should take place

A further crop of crystals may be obtained by precipitating the mother liquors with saturated ammonium sulfate and treating the precipitate obtained in this way as described under crystallization

The optical activity and specific enzymatic activity of the chymo trypsin remain constant through at least three fractional crystalliza tions as shown in Table VI The chymo trypsin crystals are shown in Fig 5

Change in Activity with Decrease in Native Protein

When the chymo trypsin protein is denatured in M/10 hydrochloric acid the decrease in activity is proportional to the decrease in native
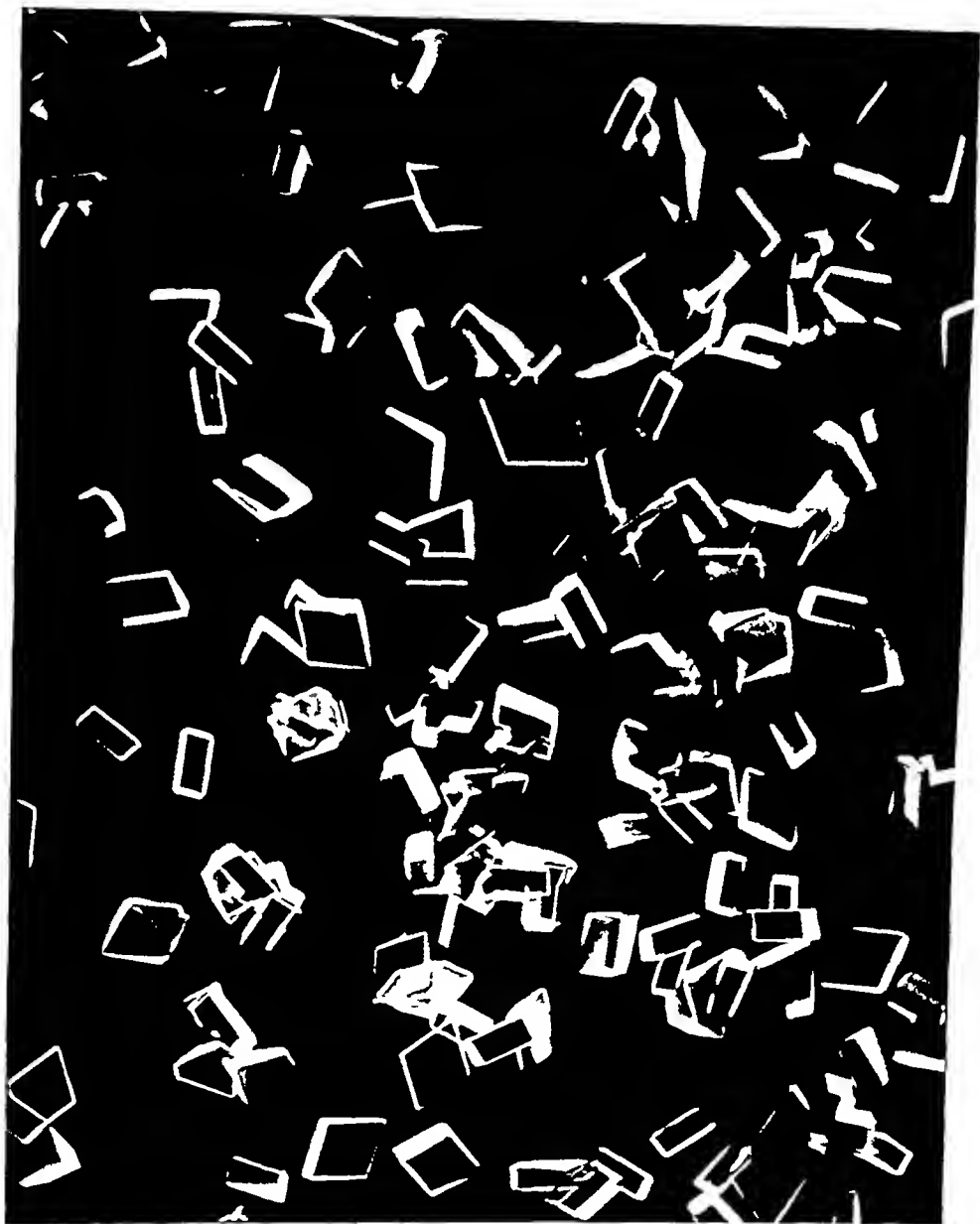
FIG 5 Chymo-trypsin

protein concentration, as shown in Table VII   The per cent loss in
activity under these conditions is the same when measured either by
digestion of hemoglobin or by rennet activity (Table VIII)   This
indicates that the hemoglobin digestion and rennet action are due to

TABLE VI

Fractional Crystallization of Chymo Trypsin

No. of times crystallized	Optical activity $M/10$ acetic acid 25 C. $[\alpha]_D^{25C}$ mg protein nitrogen	Specific activity/mg. protein nitrogen			
		Hemoglobin $[T\ U]_{mg\ protein\ nitrogen}^{Hb}$	Gelatin V $[T\ U]_{mg\ protein\ nitrogen}^{Gel.\ V}$	Casein S. $[T\ U]_{mg\ protein\ nitrogen}^{Cas.\ S.}$	Casein F $[T\ U]_{mg\ protein\ nitrogen}^{Cas.\ F}$
1	0 386	0 039	11 3	0 98	0 077
2	0 416	0 037	12 0	1 05	0 079
3	0 380	0 038	10 7	1 01	0 073

TABLE VII

Changes in Activity and Native Protein of Chymo Trypsin Solutions in $N/10$ Hydrochloric Acid, 20 C

10 ml chymo-trypsin solution (0 8 mg protein nitrogen/ml) + 10 ml $N/5$ hydrochloric acid, 20 C   2 ml samples taken and added to 2 0 ml $N/10$ sodium hydroxide, solution (No 2)   Activity by hemoglobin method

Native protein nitrogen 2 ml (No 2) + 2 ml. 2 M sodium chloride in $M/200$ hydrochloric acid   Precipitate = denatured protein, filter   Protein in filtrate determined by turbidity method

Time at 20°C *hrs*	0	1 3	4	7	16
$[T\ U]_{ml}^{Hb}$	0 015	0 011	0 081	0 0052	0 0016
Native protein N/ml , *mg*	0 390	0 256	0 172	0 144	0 031
$[T\ U]_{mg\ protein\ nitrogen}^{Hb}$	0 038	0 043	0 047	0 036	0 052

TABLE VIII

Inactivation of Chymo Trypsin Solution in $M/10$ Hydrochloric Acid 25 C Measured by Hemoglobin Method and Rennet Action

2 ml. chymo-trypsin solution (1 mg protein nitrogen/ml.) + 8 ml. $M/8$ hydrochloric acid, 25 C   Analyzed for activity by hemoglobin and rennet methods

Time at 25 C. *min*		0	20	60	240
Per cent activity by	Hemoglobin	[100]	78	55	33
	Rennet	[100]	79	60	30

the same molecule and confirms the results of the activation experiment described in Table V

If the chymo-trypsin is heated to 100°C in M/400 hydrochloric acid it is very rapidly and completely inactivated with the formation of denatured protein as shown by the fact that the protein is com-

TABLE IX

Reversible and Irreversible Inactivation of Chymo-Trypsin at 100°C

10 ml chymo-trypsin solution (0 33 mg protein nitrogen/ml) in M/400 hydrochloric acid, immersed in boiling water (No 1)

Activity and native protein in hot solution 1 ml (No 1) + 4 ml hot M/400 hydrochloric acid, 2 ml of this solution + 2 ml 2 M sodium chloride (20°C) filter, activity and protein nitrogen determined on filtrate

Activity and native protein after reversal by cooling 1 ml (No 1) + 4 ml cold M/400 hydrochloric acid, 10 minutes 20°C 2 ml + 2 ml 2 M sodium chloride Activity and protein nitrogen determined on filtrate

Time at 100°C , *min*	Not heated	1	5	15	30
Activity and native protein in hot solution					
Activity $[T\ U]^{Hb}_{ml}$	0 017	0	0		
Native protein nitrogen/ml , *mg*	0 33	0	0		
Per cent total inactivation	0	100	100		
Activity and native protein after reversal by cooling					
$[T\ U]^{Hb}_{ml}$	0 017		0 014	0 0084	0 0054
$[T\ U]^{Rennet}_{ml}$	1 90		1 7	1 0	0 54
Protein nitrogen/ml , *mg*	0 33		0 28	0 22	0 15
$[T\ U]^{Hb}_{mg\ protein\ nitrogen}$	0 052		0 05	0 038	0 036
$[T\ U]^{Rennet}_{mg\ protein\ nitrogen}$	5 8		6 1	4 6	3 6

pletely precipitated when the hot solution is poured into an equal volume of 2 M sodium chloride, and by the fact that the filtrate from the salt precipitate is completely inactive However, if the heated solution is cooled and allowed to stand at 20°C the solution recovers its original activity and the protein, like unheated chymo-trypsin is soluble in M/1 sodium chloride Thus, the denaturation and inacti-

vation of chymo trypsin by heat is completely reversible as has already
been shown to be true in the case of trypsin (9)   If the native protein
is assumed to be merely a carrier for an hypothetical "active group"
it is necessary to assume that the active group becomes inactive when
the protein is denatured and then becomes active again when the pro
tein reverts to the native condition   On longer heating this reversibly
inactivated and denatured form gradually changes to an irreversibly
inactivated and denatured form which does not become active and
salt soluble again on cooling and standing at 20°C (Table IX)

If chymo trypsin solutions are allowed to stand at pH 9 0 and 37°C
there is a loss in protein nitrogen paralleled by the loss in activity

TABLE X

Decrease in Protein Nitrogen and Activity in Chymo Trypsin Solutions pH 9 0, 37 C

20 ml chymo-trypsin solution (0 48 mg protein nitrogen/ml) + 20 ml м/10
borate buffer pH 9 0, activity and protein nitrogen determined

Time at 37°C. *hrs*	0	1	2	19
$[T\ U]_{ml}^{Hb} \times 10^{-4}$	1 0	0 84	0 60	0 22
Protein nitrogen/ml , *mg*	0 23	0 19	0 13	0 050
$[T\ U]_{mg}^{Hb}$ protein nitrogen	0 043	0 044	0 046	0 044

(Table X) This reaction is probably analogous to the inactivation
of trypsin in alkali (10) and is due to the formation and subsequent
hydrolysis of denatured protein.

The connection between the protein and the activity may also be
tested by pepsin digestion   The hydrolysis of the protein by pepsin
is accompanied by a corresponding decrease in activity (Table XI)

General Properties of Chymo-Trypsin

pH of Maximum Stability —Dilute solutions of the enzyme at 37°C
are most stable at pH 3 0-3 5 (Table XII)

Effect of pH on the Rate of Digestion by Chymo Trypsin —The rate
of digestion of casein by chymo-trypsin at various pH is shown in Fig
6   The pH activity curve is similar to that of trypsin (8)

Digestion of Sturin —Waldschmidt Leitz (11) has found that the

digestion of protamines by pig pancreas, previously considered as a property of "trypsin-kinase" is due partly to a separate enzyme, "protaminase," which may be separated from the proteolytic enzyme by adsorption of the latter on egg albumin These experiments were repeated with chymo-trypsin but it was not possible to separate the

TABLE XI

Decrease in Activity and Protein Nitrogen during Digestion of Chymo-Trypsin by Pepsin pH 2 0, 35°C

25 ml crystalline chymo-trypsin solution in м/100 hydrochloric acid (0 1 mg protein nitrogen/ml) + 1 ml crystalline pepsin solution (0 1 mg protein nitrogen) Analyzed for protein nitrogen and activity by hemoglobin method

Time at 35°C , *hrs*	0	0 5	1 0	2 0
$[T\ U]_{ml}^{Hb} \times 10^{-3}$	4 3	0 89	0 66	0 53
Protein nitrogen/ml , *mg*	0 09	0 025	0 015	0 011
$[T\ U]_{mg}^{Hb}$ protein nitrogen	0 048	0 036	0 044	0 048

TABLE XII

Inactivation of Chymo-Trypsin at Various pH, 37°C

5 ml chymo-trypsin (0 03 mg protein nitrogen/ml) + 5 ml buffers + toluene, 37°C Activity determined by hemoglobin method

pH Buffer	1 2 м/5 HCl	3 0 м/400 HCl	3 5 м/5 acetate	5 м/5 acetate	6 м/5 PO₄	8 м/5 PO₄
Time at 37°C	$[T\ U]_{ml}^{Hb} \times 10^{-4}$					
days						
0	7 1	7 1	7 1	7 1	7 1	7 1
1	0	7 0	7 1	6 6	5 7	2 7
5	0	6 0	5 7	5 1	2 5	0

protaminase activity from the proteinase activity by this method The per cent activity removed by the egg albumin was the same as determined by either casein, hemoglobin, or sturin digestion (Table XIII)

This result confirms Waldschmidt-Leitz's statement that hydrolysis of sturin is caused by the "proteinase" and not by "protaminase" (12)[3]

Digestion of Peptides —The chymo trypsin has no measurable effect upon the hydrolysis of any of the di- and polypeptides available (Table XIV)
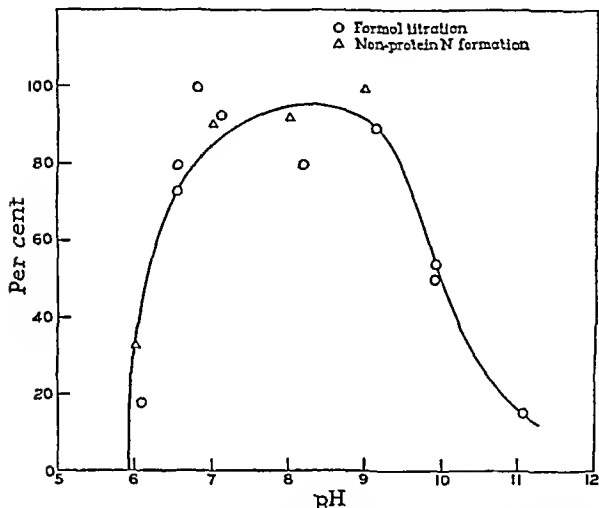
FIG 6 Digestion of casein at various pH by chymo-trypsin Casein solution 5 gm Hammarsten's casein plus 95 ml $M/10$ phosphate buffer plus 5 ml various concentrations sodium hydroxide pH determined by hydrogen electrode Chymo-trypsin solution 0 02 mg protein nitrogen/ml 1 ml chymo-trypsin plus 5 ml. casein, 35 C Digestion determined by formol titration or non protein nitrogen

Extent of Hydrolysis of Casein by Chymo-Trypsin —Casein is hydrolyzed more completely by chymo trypsin than by crystalline trypsin (8) but the hydrolysis by the two enzymes occurs at different link

[3] The writers are indebted to Professor Waldschmidt Leitz for calling their attention to this result

ages   This is shown by the fact that addition of trypsin to casein previously hydrolyzed with chymo-trypsin (Fig 7), or of chymo-

<div align="center">TABLE XIII</div>

<div align="center">*Effect of Precipitation with Acetone and Egg Albumin on Digestion of Casein, Hemoglobin, and Sturin by Chymo-Trypsin*</div>

Chymo-trypsin solution (0 3 mg protein nitrogen/ml) in м/20 pH 5 0 acetate buffer

Egg albumin (Kahlbaum) various concentrations in м/20 pH 5 0 acetate buffer

1 ml egg albumin added to 4 ml chymo-trypsin, 30°C , 1 hour   3 ml acetone added, centrifuged 10 minutes   Supernatant in vacuum desiccator 6 hours, then at 6°C 3 days, made up to 8 ml with м/20 pH 5 0 acetate buffer and activity measured on casein, hemoglobin, and sturin

Sturin activity determination 2 ml enzyme + 10 ml 2 5 per cent sturin pH 7 6, 35°C   2 ml samples added to excess м/50 sodium hydroxide   1 ml formaldehyde and phenolphthalein added and titrated to pH 9 0 with м/50 hydrochloric acid

Concentration egg albumin, *per cent*		0	0 5	1 0	1 5	2 0	3 0
Per cent original activity remaining in supernatant as determined by	Casein	100	84	60	41	30	15
	Hemoglobin	100	83	58	40	28	15
	Sturin	100		64		32	

<div align="center">TABLE XIV</div>

<div align="center">*Hydrolysis Dipeptides and Polypeptides by Chymo-Trypsin*</div>

Dipeptides 10 ml м/25 solution in м/10 phosphate buffer + 0 5 ml chymo-trypsin solution (0 25 mg protein nitrogen/ml) pH adjusted to 7 6   2 ml titrated + м/50 sodium hydroxide and formaldehyde

Tetrapeptides 10 ml м/100 solution   Digestion mixture made up as above

Polypeptides [4] The following—glycyl-*l*-tryptophane, glycyl-alanine, glycyl *l*-tyrosine, glycyl-glycine, *d*-leucyl-glycine, *d*-leucyl-glycyl-glycine, chloracetyl-*l*-tyrosine, glycyl aspartic acid, chloracetyl-*l* leucine, tri *l*-alanyl-*l* alanine, tetra *dl* alanyl-*dl*-alanine, and pentaglycyl-glycine   Increase in formol titration for 2 mL after 3 days at 35°C was less than 0 15 ml м/50 sodium hydroxide

trypsin to casein previously hydrolyzed with trypsin, causes a marked increase in hydrolysis (Fig 8)

[4] The writers are indebted to Professor Emil Abderhalden for most of the polypeptides
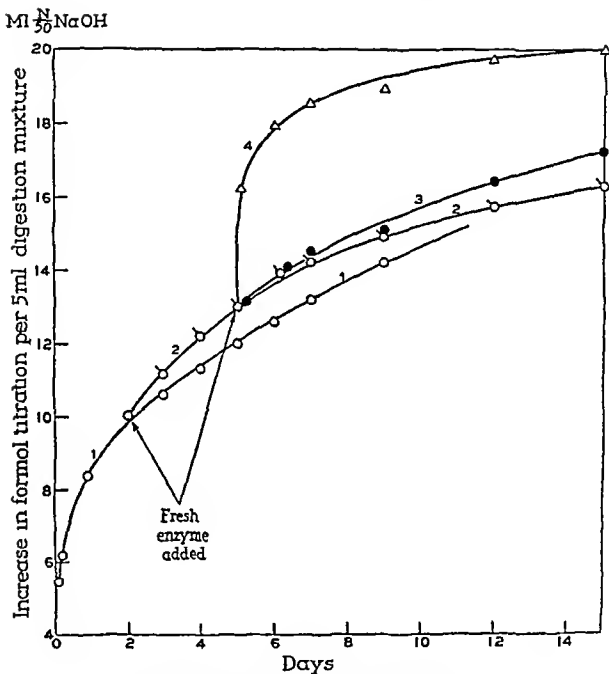
FIG 7 Digestion of casein by chymo-trypsin followed by trypsin

100 ml { 5 per cent casein pH 7 6 (M/10 phosphate buffer)
0 08 mg chymo-trypsin nitrogen/ml. ⟝ No 1

After 2 days 0 08 mg chymo trypsin nitrogen/ml added to 75 ml No 1
(Total chymo-trypsin concentration 0 16 mg nitrogen/ml) ⟝ No 2

After 5 days 0 08 mg chymo-trypsin nitrogen/ml added to 25 ml No 2
(Total chymo-trypsin concentration 0 24 mg nitrogen/ml) ⟝ No 3

After 5 days 0 08 mg crystalline trypsin nitrogen/ml added to 25 ml No 2
(Total enzyme concentration 0 16 mg chymo trypsin nitrogen/ml.
plus 0 08 mg trypsin nitrogen/ml) ⟝ No 4

Digestion determined by formol titration.

$MI\frac{N}{50}NaOH$
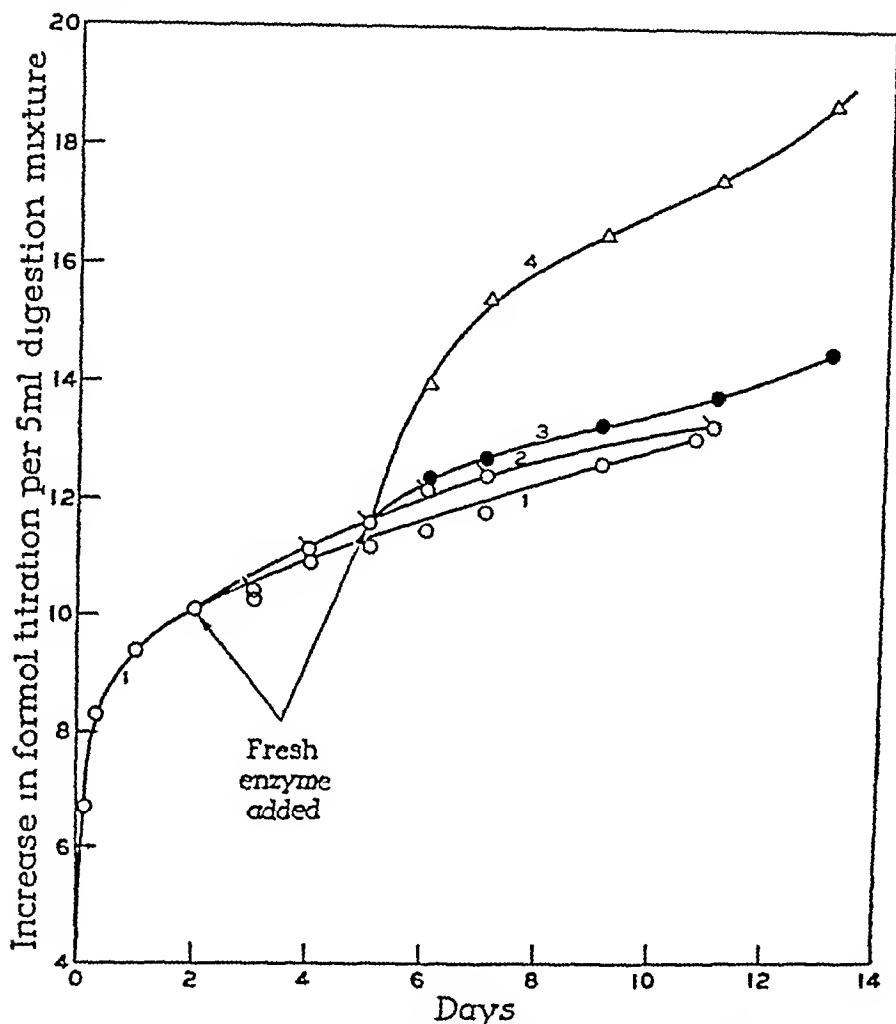
Fig 8 Digestion of casein by trypsin followed by chymo-trypsin

$100 \text{ ml} \begin{cases} 5 \text{ per cent casein pH 7 6 (m/10 phosphate)} \\ 0\ 08 \text{ mg trypsin nitrogen/ml} \end{cases}$ = No 1

After 2 days 0 08 mg crystalline trypsin nitrogen/ml added to 75 ml
No 1
   (Total trypsin concentration 0 16 mg nitrogen/ml)   = No 2
After 5 days 0 08 mg crystalline trypsin nitrogen/ml added to 25 ml
No 2
   (Total trypsin concentration 0 24 mg nitrogen/ml)   = No 3
After 5 days 0 08 mg chymo-trypsin nitrogen/ml added to 25 ml No 2
   (Total enzyme concentration 0 16 mg trypsin nitrogen/ml plus 0 08
   mg chymo trypsin nitrogen/mL)   = No 4
Digestion determined by formol titration

TABLE XV

Summary of the Properties of Chymo Trypsinogen, Chymo-Trypsin, and Crystalline Trypsin

		Chymo-trypsinogen	Chymo-trypsin	Trypsin
Crystalline form		Long square prisms	Rhombo-hedrons	Short prisms
Elementary analysis per cent dry weight	Carbon	50 6	50 0	50 0
	Hydrogen	7 0	7 06	7 1
	Nitrogen	15 8	15 5	15 0
	Chlorine	0 17	0 16	2 85
	Sulfur	1 9	1 85	1 1
	Phosphorus	0	0	0
	Ash	0 1	0 12	1 0
Amino nitrogen as per cent total nitrogen	By formol	4 7	6 0	9 3
	By Van Slyke	4 75	6 0	
Tyrosine + tryptophane equivalent milli equivalents/mg total nitrogen		$2 5 \times 10^{-2}$	$2 7 \times 10^{-2}$	3×10^{-2}
Optical activity, 25 C		In $\mu/10$ acetic acid		pH 4 0 in 0 25 sat ammonium sulfate
$[\alpha]D$ line, per mg nitrogen		−0 48	−0 40	−0 27
Solubility in distilled water		Slight	Very soluble	Very soluble
		In $\mu/2$ K$_2$SO$_4$ pH 4 0	$\mu/10$ acetate	In 0 5 sat MgSO$_4$
Diffusion coefficient, 6°C cm²/day	By nitrogen	0 039	0 037	0 023
	By hemoglobin		0 039	
	By rennet		0 037	
Molecular volume from diffusion coefficient cm²/mole		52 000	52 000	65 000
Molecular weight from osmotic pressure		36,000 (32,000)	41,000	36,500
Hydration gm water/gm protein, from osmotic pressure and diffusion coefficient.		0 7	0 5	0 8
By viscosity		0	0 1	0 6
Isoelectric point from cataphoresis of collodion particles		5 0	5 4	7–8
Specific activity [T U] per mg protein nitrogen	Substrate			
	Hemoglobin	$<1 \times 10^{-5}$	0 04	0 17
	Casein, sol	$<1 \times 10^{-5}$	1 0	2 4
	Casein F	$<0 01$	0 08	0 18
	Gelatin V	$<0 1$	12 0	100
	Rennet	$<0 01$	8 5	$<0 1$
	Clot blood	$<2 0$	$<2 0$	1500
	Sturin F	$<2 \times 10^{-4}$	0 018	0 63
pH optimum for digestion casein			8–9	8–9
Total digestion casein, ml. $\mu/50$ sodium hydroxide/5 ml 5 per cent casein			17	9–11

Isoelectric Point —The isoelectric point was determined by measuring (21) the rate of migration of collodion particles immersed in the enzyme solutions at various pH   This method shows an isoelectric point for chymo-trypsin at pH 5 4 and for chymo-trypsinogen at 5 0

Diffusion Coefficient (13) —The diffusion coefficient of chymo-trypsin was determined by measuring the rate of diffusion of the protein nitrogen and the activity   Both methods gave a diffusion coefficient of 0 037 cm ^{2}/day for chymo-trypsin   This result shows that the active molecule diffuses at the same rate as does the protein and furnishes additional evidence for the identity of the two molecules

The diffusion coefficient for chymo-trypsinogen was measured by the nitrogen only and is 0 039 cm ^{2}/day   The molecular volume corresponding to this diffusion coefficient is about 52,000 cm ^{3} on the assumption that the molecules are spheres

The molecular weight of both proteins from osmotic pressure measurements is about 40,000 so that from these measurements the proteins are hydrated to the extent of about $\frac{1}{2}$ gm of water per gm protein   Viscosity measurements, however, give much lower hydrations

The general properties of the various preparations are summarized in Table XV

Methods

1 Amylase Activity (14) —1 ml 0 5 per cent starch in M/10 phosphate buffer pH 6 8 and M/200 sodium chloride plus 0 2 ml enzyme solution of various concentrations   Left at 35 5°C for 20 minutes and 2 drops of 0 01 N iodine solution added   Dilution of enzyme which gives purple wine color taken as positive for amylase

2 Lipase —Surface tension method of Rona and Michaelis as well as the modified method of Willstatter and Memmen (15) were used   It was found very convenient to use the surface tension apparatus of du Nouy instead of a stalagmometer for the measurements of the rate of change of surface tension

3 Blood Clotting (16) —3 volumes of cattle blood plus 1 volume 28 per cent MgSO$_4$, centrifuged   3 ml supernatant plasma plus 6 ml   M/1 sodium chloride plus 2 ml M/200 CaCl$_2$ made up to 30 ml with water   2 ml of dilute plasma plus 1 ml of various dilutions of enzyme solution in M/400 hydrochloric acid mixed in 50 ml test tubes   Left in cold room for 24 hours   Minimum concentration of enzyme required to clot determined   [T U] $\frac{Clot}{mg}$ = ml dilute plasma clotted b, 1 mg nitrogen in 24 hours, 5°C

4 Proteolytic Activity —Northrop and Kunitz (17), Anson and Mirsky, hemoglobin method (18)

5 *Rennet Action* —Kunitz (19)

6 *Protein Nitrogen Precipitation* —1 ml protein solution plus 1 ml 5 per cent trichloracetic acid, heated for 5 minutes at 75 C

(a) Micro Kjeldahl method for solutions containing 1 0 to 0 3 mg protein nitrogen/ml

(b) Photoelectric turbidity method for solutions containing less than 0 3 mg protein nitrogen/ml.

The precipitate was kept in uniform suspension by rotating the cell during the photoelectric turbidity measurement as suggested by Dr A K Parpart of Princeton University

Most of the measurements reported in this paper were made by Miss Margaret R McDonald

SUMMARY

A new crystalline protein, chymo trypsinogen, has been isolated from acid extracts of fresh cattle pancreas This protein is not an enzyme but is transformed by minute amounts of trypsin into an active proteolytic enzyme called chymo trypsin The chymo trypsin has also been obtained in crystalline form

The chymo trypsinogen cannot be activated by enterokinase, pepsin, inactive trypsin, or calcium chloride There is an extremely slow spontaneous activation upon standing in solution

The activation of chymo trypsinogen by trypsin follows the course of a monomolecular reaction the velocity constant of which is proportional to the trypsin concentration and independent of the chymo trypsinogen concentration. The rate of activation is a maximum at pH 7 0–8 0 Activation is accompanied by an increase of six primary amino groups per mole but no split products could be found, indicating that the activation consists in an intramolecular rearrangement There is a slight change in optical activity but no change in molecular weight

The physical and chemical properties of both proteins are constant through a series of fractional crystallizations

The activity of chymo trypsin decreases in proportion to the destruction of the native protein by pepsin digestion or denaturation by heat or acid

Chymo trypsin has powerful milk clotting power but does not clot blood plasma and differs qualitatively in this respect from the crystal

line trypsin previously reported   It hydrolyzes sturin, casein, gelatin, and hemoglobin more slowly than does crystalline trypsin but the hydrolysis of casein is carried much further   The hydrolysis takes place at different linkages from those attacked by trypsin   The optimum pH for the digestion of casein is about 8 0–9 0   It does not hydrolyze any of a series of dipeptides or polypeptides tested

Several chemical and physical properties of both proteins have been determined

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A METHOD FOR DETERMINING THE RENNET ACTIVITY OF CHYMO TRYPSIN

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When pepsin is added to milk a gradual rise in viscosity takes place until the milk begins to clot A quantitative expression for the rennet activity of pepsin can thus be given in terms of the increase in viscosity of the milk solution (1) The viscosity method is sufficiently precise and can be conveniently used in all cases of clotting of milk or blood where the visible clotting follows a gradual increase in the viscosity of the fluid

When chymo trypsin is added to milk a slight, gradual drop in the viscosity of the milk takes place, there is no increase in viscosity until the beginning of clotting when the viscosity rises very rapidly and the solution suddenly sets to a solid gel This is shown in Fig 1

The abrupt solidification of milk by chymo trypsin enables the exact time of clotting to be conveniently determined The various methods described in the literature for measuring the clotting of milk or blood (2) are either very complicated and time consuming or are not precise The following method, used during a long series of investigations, was found to be quite accurate and very simple in operation The method is based on the fact that when a concentrated dried milk powder solution, to which chymo trypsin has been added, is allowed to flow slowly through a narrow tube the uniform flow of the milk is either brought to a sudden stop when the milk clots or, if the clot is soft, a definite mark of curd is left on the walls of the tube even after the clotted milk has continued to flow slowly through the tube The volume of milk which escapes from the tube before clotting occurs can thus be determined If, in addition, the rate of flow of the milk in the tube is known then the time required for the milk to clot can be readily calculated
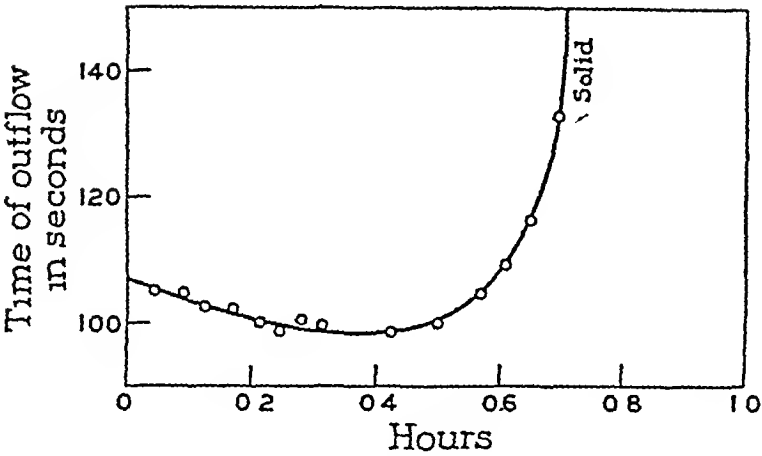
Fig 1 Effect of chymo-trypsin on viscosity of 10 per cent solution of Klim milk, pH 5 0 at 35 5°C
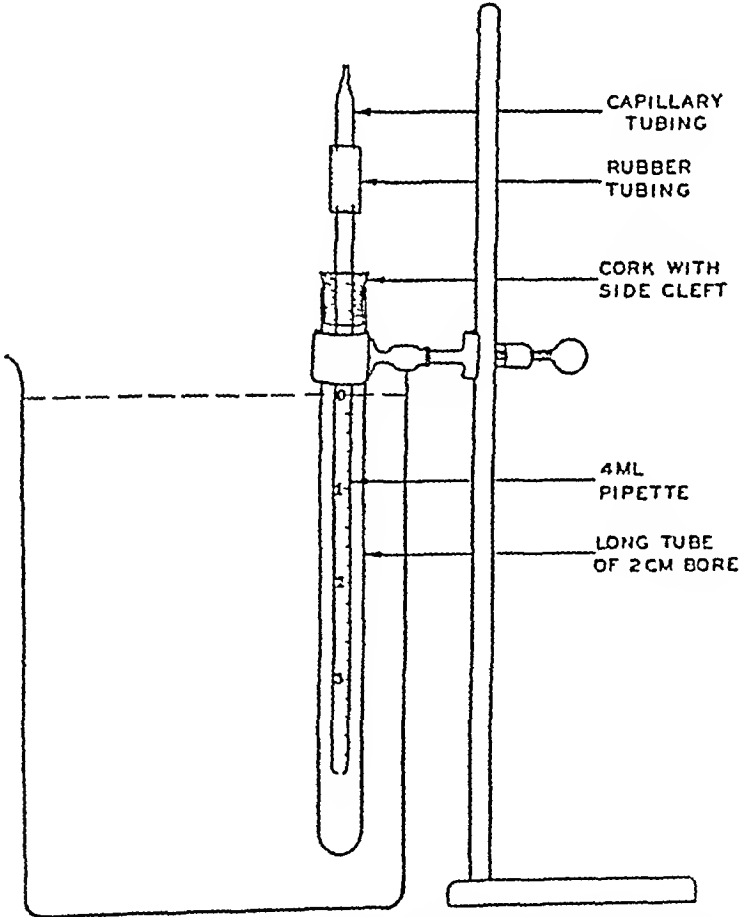
Fig 2 Apparatus for measuring the rennet activity of chymo-trypsin

Apparatus

The apparatus used is similar to that described by Heubner and Rona (3) for blood clotting but is much simpler in construction It consists of the following parts, as shown in Fig 2

1 A straight pipette of 4 ml content, graduated to 0 1 ml It is made from an ordinary 5 ml measuring pipette by cutting off the tip at the 4 1 ml mark and then fusing the end to a bore of about 1 mm in diameter The graduations are, as usual, from 0 downwards The marks are rubbed in with a black glass pencil to make them more conspicuous when the pipette is filled with milk

2 A glass tube about 30 cm long and 2 cm inner bore The tube is clamped in a vertical position in a constant temperature bath with transparent walls

3 A number of short pieces of glass tubing about 3 mm in diameter drawn out to very fine capillaries

Milk Solution

20 gm Klim powdered milk is ground up gradually to a creamy paste in a large mortar by the addition of increasing amounts of distilled water, then washed into a 100 ml volumetric flask containing 10 ml of M/1 sterile acetate buffer pH 5 0 The solution is made up to mark with water and filtered through gauze The milk, after addition of toluene can be stored for 2 or 3 weeks at 5°C without any significant change in its behavior

Operation

1 ml enzyme in water or in M/10 acetate buffer pH 5 0 is blown into a test tube containing 10 ml milk at 35 5°C and the tube is stirred immediately A stop watch is started at the moment of addition of the enzyme The milk is drawn up to the zero mark of the 4 ml pipette by means of a short piece of rubber tubing fitted on the pipette and provided with a spring clamp When the milk has reached the zero mark the tubing is clamped and a suitable capillary tubing is inserted in the rubber tubing The spring clamp is removed from the rubber tubing while the tip of the pipette is still in the milk This brings the level of the milk slightly above the zero mark The pipette is transferred into the long glass tube

in the water bath and fixed in position   The time required for the first 0 5 ml of milk to flow out is then determined by means of a second stop-watch   The first stop-watch is stopped immediately after starting the second watch   The reading on the first watch indicates the time elapsed between the moment of mixing the enzyme with the milk and the moment when the milk passed the zero mark in the pipette during its continuous flow

The pipette is left in the bath until the milk clots   Observation is made afterwards of the height of the clotted milk in the pipette This observation can be made any time after clotting and it is unnecessary to watch the experiment   If the clot is soft it will continue to flow but there is always a distinct curd left on the wall of the pipette which shows where clotting began

The method is convenient as well as rapid   A large number of pipettes can be started one after another and left alone in the bath for final reading

It is advisable to use capillaries of such dimensions that the first 0 5 ml   requires 3 to 5 minutes to flow   Such concentrations of enzyme should be selected that clotting occurs within 10 to 20 minutes after addition of the enzyme to the milk solution

Calculations

It was mentioned above that in the case of clotting of milk by chymo-trypsin the viscosity of the milk is not changed appreciably until the moment of clotting   The rate of the flow of milk depends, then, on the size of the capillary on the top of the pipette as well as on the opening at the outflow, and is proportional to the height of the milk in the pipette, i c ,

$$-\frac{dh}{dt} = Kh \qquad \text{(Equation 1)}$$

Hence, the time required for the outflow of any volume of milk from the level h_o to level h_t is

$$t = \frac{2.3}{K} \log \frac{h_o}{h_t} \qquad \text{(Equation 2)}$$

Since the pipettes are graduated from zero downwards the original height h_o of the milk is 4 0 and the height h_t at any time t during

flowing is 4 0 minus the graduation mark on the pipette at the level of the milk   The time required for the outflow of a volume, r, of milk is therefore

$$t = \frac{2.3}{K} [\log 4\,0 - \log (4\,0 - r)] \qquad \text{(Equation 3)}$$

where r is the reading of the pipette at the point where the milk clots
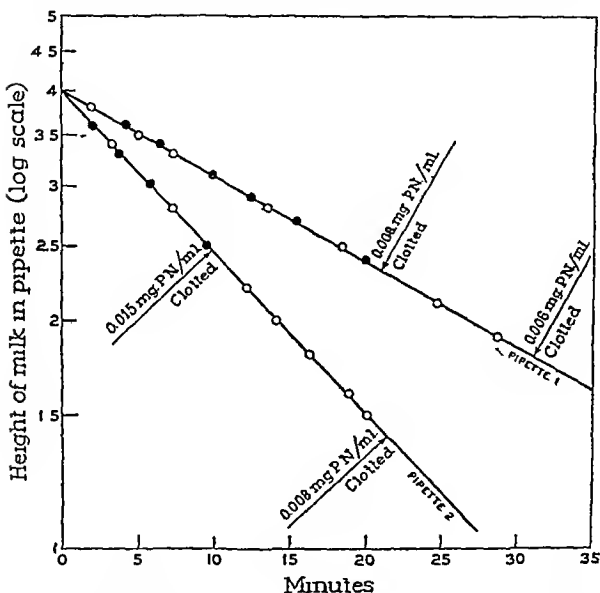
FIG 3 Flow of 18 per cent solution of Klim milk powder containing various amounts of chymo-trypsin

Equation 3 was checked by plotting on semi log paper the time curves of the flow through two pipettes of milk containing various amounts of enzyme   A large number of readings were taken in this case in order to obtain many points for the curves   The curves ob-

tained (Fig 3) are straight lines up to the very time of clotting   As the figure shows, the time of clotting of a solution of milk powder containing a definite amount of enzyme is the same for both pipettes although the rate of flow differed in the two cases [1]

The time required for clotting of the milk is calculated as follows Let l_1 equal the time in minutes read on the first stop-watch, i e , the time elapsed between the moment of mixing of the enzyme with the milk and the moment at which the milk passes through the 0 mark on the pipette.

l_2 equals the time read on the second stop-watch, i e , the time taken for the milk to drop from 0 to 0 5 ml

l_3 equals the time required for the milk to drop from the zero mark to the final clotting mark, r (The value of l_3 is calculated from Equation 3, or read off the graph, by holding a straight edge against the points $(0,4)$ and $(l_2, 3 5)$ on semi-log paper and then reading l_3 on the time coordinate which corresponds to the value of $4 - r$ on the log coordinate)   The total time required to clot the milk from the moment the enzyme is added is

$$t = l_1 + l_3$$

Definition of Rennet Unit of Activity

One unit of rennet activity $[T\ U]^{Rennet}$ is the amount of activity that causes clotting of 11 ml of 18 per cent Klim milk in $M/10$ acetate buffer pH 5 0 in 1 minute at 35 5°C   The 11 ml of mixture contains 1 ml of enzyme solution so that if t minutes are required for the milk to clot then 1 ml of the enzyme solution contains $\frac{1}{t}$ units of rennet activity   This last number, when divided by the milligrams protein nitrogen contained per milliliter of the enzyme solution, gives the specific activity of the enzyme per milligram protein nitrogen, i e ,

$$[T\ U]^{Rennet}_{mg\ pro\ en\ n\ gm} = \frac{1}{t \times mg\ protein\ nitrogen/ml}$$

Example —1 ml chymo-trypsin solution in water containing 0 015 mg protein nitrogen was added to 10 ml 20 per cent milk solution at

35 5° C   It required 1 6 minutes to draw the milk into the pipette, place the pipette in position, and for the milk to pass the zero mark.   The time consumed in this way was read from the first stop-watch.   Thus, $t_1 = 1\,6$ minutes   It required 2 7 minutes for the milk to flow from 0 to the 0 5 mark as read off the second stop-watch, i e, $t_2 = 2\,7$ minutes   The milk clotted when it reached the mark $r = 1\,55$ The corresponding value of h_t is $4\,0 - 1\,55 = 2\,45$ A straight line was drawn on semi log paper through the point $t = 0$, $h = 4\,0$, and the point $t = 2\,7$, $h = 3\,5$ The time ordinate of the point on
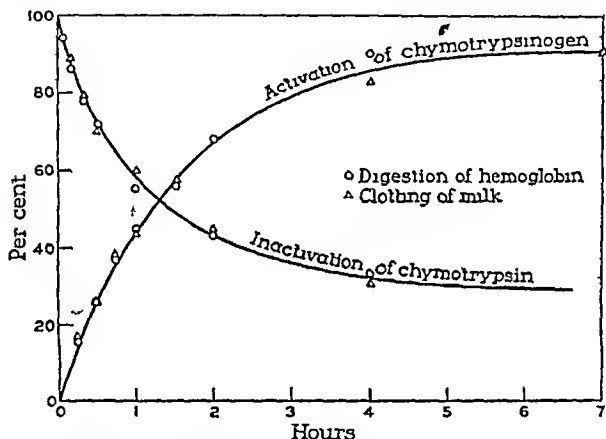
FIG 4   Activity of chymo trypsin as determined by its effect on digestion of hemoglobin and on clotting of milk.

this line corresponding to the value of $h = 2\,45$ was $t_3 = 9\,8$ minutes which equals the time required for the milk to drop from the zero mark to the clotting mark, r The total time required to clot is

$$t = t_1 + t_3 = 11\,4 \text{ minutes}$$

$$[\text{T U}]^{\text{Rennet}}_{\text{mg protein nitrogen}} = \frac{1}{11\,4 \times 0\,015} = 5.84$$

Numerous measurements of rennet activity of chymo trypsin have been made in connection with the experiments described elsewhere

(3) In all cases the precision obtained for the rennet activity of the chymo-trypsin by the method described here was of the same order of magnitude as that obtained in the measurements of the proteolytic activity of the enzyme. Fig 4 shows a curve for the rate of activation of chymo-trypsinogen by trypsin as measured by digestion of hemoglobin and by clotting of milk expressed as per cent of final activity The curve shows that the two methods of determining the amount of active chymo-trypsin produced at various times during activation give practically identical values   The same result is shown by the curve for inactivation of chymo-trypsin in M/10 hydrochloric acid at 25°C

The method was found to be applicable also to the determination of the clotting of milk by pepsin   The change in viscosity preceding clotting does not cause any significant change in the rate of flow, owing to the very large aperture of the pipette

SUMMARY

The rennet activity of chymo-trypsin (or pepsin) is conveniently measured by allowing a standard solution of milk to which chymo-trypsin has been added to flow slowly through a graduated pipette and observing the rate and distance of flow of the milk before it clots The time required for chymo-trypsin to clot milk may be calculated from these observations   The rennet activity is expressed as the reciprocal of the time in minutes required for 1 ml of enzyme solution to clot 10 ml of standard milk powder solution

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THE CONDUCTIVITIES OF AQUEOUS SOLUTIONS OF GLYCINE, d,l VALINE, AND l-ASPARAGINE*

By JOHN W MEHL and CARL L A SCHMIDT

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30 years ago, Sir James Walker (1, 2) published two papers entitled, "The theory of amphoteric electrolytes," in which he discussed the conductivities of amphoteric electrolytes and their deviations from the Ostwald dilution law in terms of the mass law Deriving the expressions for the various ion concentrations present in an ampholyte solution, he showed why the attempt to apply the dilution law to such solutions is invalid He was able to show that the conductivities calculated from the relations he had obtained were in agreement with Winkelblech's (3) data for the aminobenzoic acids

With the exception of a single value for asparagine, the figures given by Walker are not concerned with the aliphatic amino acids At the time there were no trustworthy data for the latter compounds In 1895, Emile Franke (4) reported values for α alanine up to a concentration of 0 0156 M, and in 1905, Siegfried (5) published more measurements on alanine, this time from 0 125 to 1 0 M Values at isolated concentrations are also given by Bayliss (6) More recently the conductivity of glycine has been measured by Miyamoto and Schmidt (7) In connection with the measurement of the conductivity of asparagine, Walker pointed out the fact that the only criterion for the purity of such a compound is the attainment of constant conductivity on repeated purification, and that such a result was only obtained after the asparagine had been recrystallized twenty four times Since the same precautions were not applied to the alanine and glycine used in the work mentioned above, it was thought worth while to attempt to measure the conductivities of several of the aliphatic

α-monoamino-monocarboxylic acids in order to determine whether values agreeing with those which can be calculated may be obtained, and whether such conductivity data may be used in the determination of the ionic mobilities of these amino acids   A study of the type herein reported would, it was believed, throw considerable light on the accuracy as well as the value of amino acid conductivity data

The compounds studied are glycine, *d,l*-valine, and *l*-asparagine The effect of alanine on the conductivity of KCl has also been studied in order to determine whether the alanine zwitter ion makes a contribution to the ionic atmosphere of the solution which is sufficient to lead to a reduction of the mobility of the ions which are present in such solutions

EXPERIMENTAL

The conductivity measurements were made according to the Kohlrausch method   The details of the apparatus are given by Miyamoto and Schmidt (7)

The purification of the amino acids was carried out by repeated recrystallization until a constant conductivity value was obtained   In the case of the synthetic amino acids (glycine, alanine, and valine) it was found that the addition of alcohol to the hot, saturated aqueous solution and repeated washing between crystallizations with alcohol made it possible to reach a constant purity after recrystallizing about eight times, while when water alone was used, the conductivity value was still decreasing after the tenth recrystallization. This is probably due to contamination by ammonium halides   In the case of *l*-asparagine, the use of alcohol does not speed the purification, but it greatly reduces the loss during crystallization   The asparagine finally used was recrystallized thirty-five times

Certain of the measurements on glycine and valine were carried out in a cell with platinized black electrodes and the others in a cell with grey platinum electrodes   It was found that electrodes coated with platinum black could not be used in those cells in which the asparagine measurements were made for the reason that, in the presence of platinum black, asparagine decomposes with the formation of ammonia as one of the end products   Cells with grey electrodes were used to measure the conductivities of asparagine solutions

The conductivity water had a specific conductance of from 1 01 to 1 2 × 10⁻⁶ mhos at 25°   The correction for the conductance of the solvent was made by subtracting its value from that of the total conductance

The measurements at 25° were made in a small oil bath with a temperature control of ±0 01°, those at 35° in a water bath with a control of ±0 1°, and those at 0° were made in melting ice

The conductivity data for glycine, *d l*-valine, and *l*-asparagine are given in Table I

TABLE I

The Specific Conductance of Glycine, d l-Valine and l-Asparagine

Temperature	Concentration	Specific conductance
	(a) Glycine	
degree	*mols/liter*	*mhos* $\times 10^6$
0	0 077	0 4
	0 196	1 2
	0 328	2 0
	0 512	3 0
	0 640	3 7
	0 800	4 5
	1 000	5 5
25	0 077	1 4
	0 196	3 7
	0 328	6 2
	0 512	9 5
	0 640	11 8
	0 800	14 5
	1 000	17 8
35	0 048	1 4
	0 100	2 9
	0 240	7 0
	0 400	11 5
	0 700	19 5
	1 000	27 1
	(b) d l Valine	
0	0 039	0 08
	0 098	0 24
	0 244	0 60
	0 406	1 00
	0 508	1 20
25	0 039	0 4
	0 098	1 1
	0 244	2 7
	0 420	4 5
	0 507	5 4
35	0 036	1 1
	0 061	1 6
	0 151	3 1
	0 252	4 7
	0 419	7 7

TABLE I—*Concluded*

Temperature	Concentration	Specific conductance
(c) *l*-Asparagine		
degrees	*mols/liter*	*rhos* $\times 10^4$
25	0 0334	1 6S
	0 0667	2 89
	0 1000	3 96
	0 1333	5 10
	0 1665	6 14
	0 2000	7 46

For the determination of the effect of the zwitter ion on the conductivity of a strong electrolyte, d,l-alanine and KCl were used For comparison, the effect of urea on the conductivity of the KCl was also determined   In each case the conductivity of the alanine or urea solution was determined separately and that value was subtracted from the one obtained for the solution containing KCl   The conductivity of alanine is very nearly the same as that of glycine, while that of the 0 05 molar urea solution used was $1\ 67 \times 10^{-6}$ The results are given in connection with the discussion

DISCUSSION

In Table II, the values obtained for the conductivities of certain amino acids by different workers are compared, graphical interpolation having been used when necessary   It is quite obvious that too great a reliance cannot be placed on measurements in which the conductivity of a concentrated solution approaches that of the solvent   Even when the values obtained with repeatedly purified samples give reasonable checks, it would appear that the validity of the results is still not assured   In this case it may simply mean that the amount of contaminating substance has reached a constant value   Furthermore, the nature of the proper correction to be applied for the conductivity of the solvent is in doubt, as has been noted by Washburn (8)   It is possible that the solute influences the dissociation of the impurity in such a manner that its contribution to the conductivity in the solution is different from that in the solvent alone   Under such cir-

cumstances simply subtracting the conductivity of the solvent will not be adequate, and the nature of the impurity will have to be known in order to make a valid correction As the water available had a conductivity considerably greater than that of pure water when carbon dioxide free, and since the nature of the contaminating substance is unknown, the correction as applied may be in error, and the extent of the error cannot be accurately estimated

On the other hand, when attempting to make any calculations involving conductivity measurements, one is faced with the fact that in most instances deviations from the laws of perfect solutions are probably considerable even in dilute solutions, and that the deviations to

TABLE II

Comparison of Specific Conductivity Data* Obtained by Various Workers

Compound	Concentration	Present work	Walker	Siegfried	Miyamoto and Schmidt
Asparagine	0 0625 м	2 8	6 0	—	—
Alanine	0 34 м	6 7	—	23 5	—
Glycine	1 00 м	17 8	—	34 7	50 9

* All conductivity values are × 10^6

be expected are not at present well defined For example, association of the zwitter ions may reduce their activity and thus reduce dissociation, but it is also possible that this effect may be counteracted or even superseded by the effect of the increasing dielectric constant Viscosity effects will enter, as will also other factors whose relative importance can no more certainly be evaluated However, the following calculations are suggestive of the general character of the results

Walker (1, 2) pointed out the fact that the rational formulation of the Ostwald dilution law results from expressing the mass action law for a weak electrolyte in terms of the conductances of the ions, and that it consequently cannot be expected to be applicable to an ampholyte He then proceeded to derive the expressions for the ion concentrations in an ampholyte solution, and we may do the same, using a slightly different terminology

If we let

R⁺ = the cation of the ampholyte,
R⁻ = the anion of the ampholyte,
R± = the undissociated ampholyte (taken to be predominantly in the zwitter ion form),

$$\frac{(R^-)\,(H^+)}{(R^\pm)} = K_1 = k_a \text{ (classical acid dissociation constant)}$$

$$\frac{(R^+)}{(R^\pm)\,(H^+)} = K_2 = k_b/k_w$$

These expressions assume that the activities may be taken as being numerically equal to the concentration   If, then, we neglect the concentration of OH⁻, which will be very small in appreciable concentrations of the aliphatic amino acids under consideration,

$$(H^+) + (R^+) = (R^-)$$

In all except very dilute solutions, the concentrations of R^+ and R^- with respect to the total concentration of amino acid may also be neglected, so that,

$$(R^\pm) = C$$

Where C = total concentration of amino acid   Solving for (H^+), we obtain

$$(H^+) = \sqrt{\frac{CK_1}{1 + CK_2}}$$

which is equivalent to the expression derived by Walker when the concentration of OH⁻ is neglected   When we solve for the concentrations of the amino acid ions and express the specific conductance in terms of these concentrations and the ionic conductances, there is obtained the expression

$$\bar{L} = \left[\frac{CK_1(\Lambda_{H^+} + \Lambda_F) + 2\Lambda_F C^2 K_1 K_2}{\sqrt{CK_1(1 + CK_2)}} \right] \times 10^{-3}$$

$$\Lambda_e = \frac{K_1(\Lambda_{H^+} - \Lambda_F) + 2\Lambda_F CK_1 K_2}{\sqrt{CK_1(1 + CK_2)}}$$

The dissociation constants of glycine are $K_1 = 1\,655 \times 10^{-10}$ and $K_2 = 224$ at 25° (9) Using the data of McBain and Dawson (10) for the diffusion of glycine, the mobility of the zwitter ion is about 40 7 which corresponds to a limiting ion conductance of 39 2 This agrees well with the limiting ion conductance of the acetate ion, viz , 40 87 (11) when the introduction of the $-NH_2$ group is considered, and the mobility of the glycine ion is taken to be the same as that of the zwitter ion In Table III, the values for the molar conductances calculated with the aid of these data are compared with the values obtained by direct measurement

TABLE III

Calculated and Determined Molar Conductance* of Glycine and l Asparagine

Concentration	Glycine		l-Asparagine	
	Calculated	Determined	Calculated	Determined
mols/liter				
0 05	20 2	18	46 0	46 1
0 10	18 0	19	35 5	40 0
0 20	16 5	19 4	30 4	37 0
0 40	15 6	19 0		
0 60	15 4	18 4		
0 80	15 4	18 1		

* All values given in Table are $\times 10^3$

For *l*-asparagine, the limiting ion conductance of the amino acid ion is taken to be the same as that found for the aspartate ion (7), which is 27 75 at 25° The dissociation constants used are $K_1 = 1\,38 \times 10^{-9}$ and $K_2 = 148$ (12) It will be seen that the agreement is satisfactory only in a qualitative way, and that the deviations with increasing concentrations are in the opposite direction from that which might be expected

Proceeding in the converse manner, we may attempt to calculate the mobility of the valine ion using the constants $K_1 = 2\,4 \times 10^{-10}$ and $K_2 = 209$ (14), and the value of $\Lambda_e = 11\,1 \times 10^{-3}$ at 0 075 molar and 25° In this way a value of 14 is obtained which is quite unsatisfactory when compared with the limiting conductances of valerate and cap

roate ions which are given as 31 and 29[1] respectively   This corresponds to a deviation from theory in the opposite direction from that which is shown by glycine and asparagine

In view of the influences which have already been mentioned as possibly being active in these solutions, it is too much to expect that any simple theory should suffice to predict the conductivities at these concentrations   Furthermore, the difficulties involved in making the measurements preclude the possibility of attributing the disagreement between the predicted and the determined conductivity values entirely to theoretical limitations   In so far as the general problem of the conductivity of aliphatic monoamino-monocarboxylic acids is concerned, it must be concluded that neither the conductivity of the salts nor of the free acids can be used in the calculation of mobilities   In the case of the monoamino-monocarboxylic acids themselves, the conductivity values are too low to be trustworthy, while in the case of their salts, too great a part of the current is carried by the hydrogen ion and anion, or hydroxyl ion and cation

In the case of certain other amino acids, the situation is more satisfactory   It has already been shown by Miyamoto and Schmidt (7) that the mobilities of the ions of the dicarboxylic amino acids may be determined from the conductivity measurements of their salts   They also point out that the dicarboxylic amino acids themselves do not obey Ostwald's dilution law, although there is apparently an approach to a constant value in the very lowest concentrations   Walker (1) has pointed out that, in the case of ampholytes which are either predominantly basic or acidic, this is to be expected   The tendency of the dicarboxylic amino acids to obey the Ostwald law is apparently due to the predominating influence of the distal carboxyl group (though this is actually the weaker carboxyl group)   While the dilution law is still not strictly applicable to these cases, measurements can be made in sufficiently dilute solutions so that a simplified theoretical formulation can be applied to the results and the conductivity data used in the calculation of the dissociation constants

For the following considerations, it will be assumed that the zwitter ion is formed predominantly between the amino group and the carboxyl

group closest to it (17, 18) The expressions for the dissociation of the dicarboxylic amino acids may be written in the following manner

$$\frac{(R^-)(H^+)}{(R^\pm)} = K_1 \qquad\qquad R^- = {}^-OOC\text{---}R\begin{smallmatrix}\diagup NH_3^+\\\diagdown COO^-\end{smallmatrix}$$

$$\frac{(R^+)}{(R^\pm)(H^+)} = K_2 \qquad\qquad R^\pm = HOOC\text{---}R\begin{smallmatrix}\diagup NH_3^+\\\diagdown COO^-\end{smallmatrix}$$

$$\frac{(R^-)(H^+)}{(R^-)} = K_3 \qquad\qquad R^+ = HOOC\text{---}R\begin{smallmatrix}\diagup NH_3^+\\\diagdown COOH\end{smallmatrix}$$
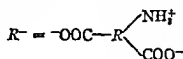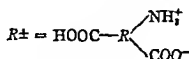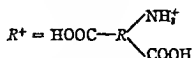
While these expressions are not complete, they should be adequate for the present purpose It will also be assumed that the effect of the third dissociation may be neglected, and no attempt will be made to correct for activities or changes in mobility in more concentrated solutions Under such conditions the following expressions are obtained

$$K_1(H^+)^2 + (CK_2 + 1)(H^+)^2 + K_1(H^+) - CK_1 = 0 \tag{1}$$

$$(R^\pm) = \frac{C}{1 + K_1/(H^+) + K_2(H^+)} \tag{2}$$

where C is the total concentration of the amino acid

Since the conductance of these solutions is largely due to the hydrogen ions in the lower concentrations, a first approximation of the hydrogen ion concentration can be made by dividing the specific conductivity by the sum of the mobilities of the hydrogen and amino acid ions (since there will be *at least* one amino acid ion for each hydrogen ion), and multiplying by 10^3 If this value is then substituted in Equation 1 for the two lowest concentrations, the equations may be solved simultaneously for K_1 and K Having a rough approximation of the value of the constants, the corresponding approximations for $R^\pm$ and R^+ may be made At any concentration that part of the conductivity which is not due to the hydrogen ion and an equal concentration of R^-, will be due to the concentration of R^+ present and an equal concentration of R^- If then, having the approximate value for the concentration of R^+, it is multiplied by the limiting conductance

value of this ion, the proper factor, and the resultant product is doubled, a correction to be subtracted from the experimental conductance is obtained in order to make a second approximation of (H^+) By such a series of approximations it is finally possible to arrive at values for K_1 and K_2 which give a satisfactory agreement with the conductivity data at lower concentrations

In this manner the values $K_1 = 4\ 45 \times 10^{-5}$ and $K_2 = 115$ were obtained for glutamic acid at 25°, and $K_1 = 1\ 24 \times 10^{-4}$ and $K_2 = 155$

TABLE IV

The Determined and Calculated Specific Conductance of Aspartic and Glutamic Acid

Concentration	Aspartic acid		Glutamic acid	
	Specific conductance		Specific conductance	
	Calculated	Determined*	Calculated	Determined*
mols/liter	*mhos $\times 10^5$*	*mhos $\times 10^5$*	*mhos $\times 10^5$*	*mhos $\times 10^5$*
0 0001	2 46	2 45	1 80	1 78
0 0002	3 98	3 94	2 77	2 76
0 0005	7 08	7 05	4 69	4 65
0 001	10 51	10 67	6 80	6 64
0 002	14 78	15 61	9 48	9 46
0 005	23 5	24 3	15 2	14 3
0 01	29 5	32 7	18 8	18 9
0 02	38 5	43 4	24 2	25 1

* Only the data of Miyamoto and Schmidt have been considered in making these calculations, so that, while the values found are comparable with those which have been calculated, any error in the conductivity measurements will introduce a corresponding error in the values calculated for the dissociation constants

for aspartic acid at the same temperature   From the figures given in Table IV it will be seen that the agreement between the experimental and calculated values for the specific conductances of aspartic and glutamic acid is quite good in the lower concentrations and that the deviation in higher concentrations is not greater than might reasonably be expected   The accuracy of the values for K_1 should be quite good, and ought not to deviate more than 5 per cent from the true constant   The accuracy with which K_2 may be calculated is considerably less since its effect is not so great as that of K_1, particularly in the

lower concentrations where the results should be most dependable
The values for K may be 20 per cent in error

In Table V, the dissociation constants for the carboxyl groups of
glutamic and aspartic acids, glycine, and asparagine (calculated in
accordance with the zwitter ion hypothesis) are compared with the
dissociation constants of glutaric, succinic, and acetic acids It will
be seen that these constants are in accord with the supposition that the
zwitter ion in the two dicarboxylic amino acids is formed with the
carboxyl group proximal to the amino group, that the amide group in
asparagine is on the distal carboxyl group, and that the effect of the
amino group on the dissociation of the carboxyl group in all three

TABLE V

The Acid Dissociation Constants of Certain Substances

Substance	K_{A_1}	K_{A_2}	Reference
Glycine	$4\ 47 \times 10^{-3}$	—	(9)
Acetic acid	$1\ 753 \times 10^{-5}$	—	(11)
Aspartic acid	$6\ 45 \times 10^{-3}$	$1\ 24 \times 10^{-4}$	Present work
Succinic acid	$6\ 3 \times 10^{-5}$	$1\ 5 \times 10^{-6}$	(19)
Asparagine	$6\ 75 \times 10^{-3}$	—	(12)
Glutamic acid	$8\ 7 \times 10^{-3}$	$4\ 45 \times 10^{-5}$	Present work
Glutaric acid	$4\ 8 \times 10^{-5}$	$3\ 2 \times 10^{-6}$	(19)

cases is of a comparable magnitude when the amino group is in the
alpha position, and decreases with increasing removal

It must be recognized that the constants given above for aspartic
and glutamic acid are not in good agreement with the values obtained
by electrometric titrations, though they are not less divergent than
values which have been obtained by different workers (13) From the
above it follows that the apparent dissociation constants of hydroxy
glutamic acid and of the diamino-monocarboxylic acids may be
similarly obtained from conductivity measurements

The Conductivity of Potassium Chloride in the Presence of Alanine

An interesting consequence of the conductivities obtained for the
monoamino monocarboxylic acids is the indication of conductivity
measurements as a means of studying the contribution of the zwitter

ion to the ionic atmosphere   By this method any complications due to effects of the electrolyte on the amino acid are obviated because of the very small conductivity of the amino acid relative to that of a strong electrolyte

Only a few such measurements have been made, but they serve to indicate the possibilities, and show quite definitely that alanine has no effect on the mobility of ions which is comparable to that of an electrolyte

The equivalent conductances of several concentrations of KCl in 0 05 M and 0 1 M alanine were determined and plotted against the square root of the KCl concentration in order to extrapolate to an infinitesimal KCl concentration   KCl in 0 05 M urea was studied in

TABLE VI

The Conductance Ratios of KCl in Alanine and Urea Solutions Referred to Pure Water

KCl in a solution of	$\frac{\Lambda_0}{\Lambda^\circ_{H_2O}}$ (25°)	$\frac{\nu}{\nu_{H_2O}}$ (18°)
0 05 M Alanine	0 995	0 99
0 05 M Urea	1 00	1 00
0 10 M Alanine	0 984	0 98

the same manner   In Table VI the ratios of these values for limiting equivalent conductances to that in water at 25° are given, together with the relative fluidities at 18°[2]

It is readily seen that a change in the viscosity of the solution is in itself enough to account for the greater part of the effect of alanine, and that there is no effect which is at all comparable to that of an electrolyte   These results confirm those of Failey (15)

In a study of various types of diions, Simms (16) has indicated that the electrical behavior of the diion will depend upon the separation of the charges, and that for the zwitter ion type the behavior where the separation is small should approach that of a neutral molecule   This appears to be the situation in the case of alanine, and probably is also the case for the other alpha-amino acids   It should be of considerable interest to determine whether or not the separation of the charges in amino acids where the distance between the carboxyl and amino groups

is greater or in peptides ever becomes great enough so that there is a resultant external field

SUMMARY

1 The conductivities of aqueous solutions of glycine, d,l valine, and l asparagine have been determined, and comparisons have been made with similar data reported in the literature

2 On the basis of certain theoretical considerations, calculations of the expected conductivities of aqueous solutions of glycine, asparagine, aspartic acid, and glutamic acid have been made and these data have been compared with similar data obtained experimentally

3 The dissociation constants of the carboxyl groups of aspartic acid and glutamic acid have been calculated from conductivity data

4 It is shown that alanine has no effect on the ionic atmosphere of solutions of potassium chloride

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OSMOTIC RELATIONSHIPS IN THE HEN'S EGG AS DETERMINED BY RELATIVE VAPOR PRESSURES

By J. M. JOHLIN

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The present paper contains data obtained by determining relative differences in the vapor pressures of the yolk and white of the hen's egg These data support previous conclusions that the yolk of the newly laid egg has a greater osmotic pressure than the white

In a previous paper the writer (1) presented freezing point data which supported the conclusion that the yolk has the greater osmotic pressure These results are contrary to those of Howard (2) who by the use of the writer's (3) freezing point method found the osmotic pressure of yolk and white to be the same Howard's variations were due to an altered technique in the use of this method That this altered technique in the manner of determining the freezing point of a highly viscous substance such as egg yolk gives freezing point values which are too high was shown by the data of the writer's (1) previous paper

Some of the freezing point determinations have since been repeated and it was found as before that if vigorous stirring, during the freezing point determinations, such as Howard insists upon, is interrupted, the temperature recorded by the thermometer drops immediately and at a faster rate than could be brought about by the abstraction of heat by the cooling bath This sudden temperature drop can only be due to the fact that the thermometer becomes heated by the stirring (which is carried out by means of the thermometer) and records a temperature which is higher than that of the egg yolk not in immediate contact with it. If upon reaching the type of temperature plateau which Howard (2) describes, the temperature of the cooling bath is rapidly raised above that of the yolk, the thermometer will still record a drop in temperature when vigorous stirring is interrupted

Vapor pressure data published by Howard (2) also indicate that the osmotic pressures of yolk and white are the same Baldes (4) has recently reconfirmed the existence of a difference in vapor pressure between yolk and white and has shown that there is a gradient of osmotic pressure in the yolk from low near the surface to high inside

In the present experiments yolk and white were exposed to the same atmosphere and allowed to progress toward an isosmotic state by the distillation of water from one to the other, with the use of air as the intervening "membrane "
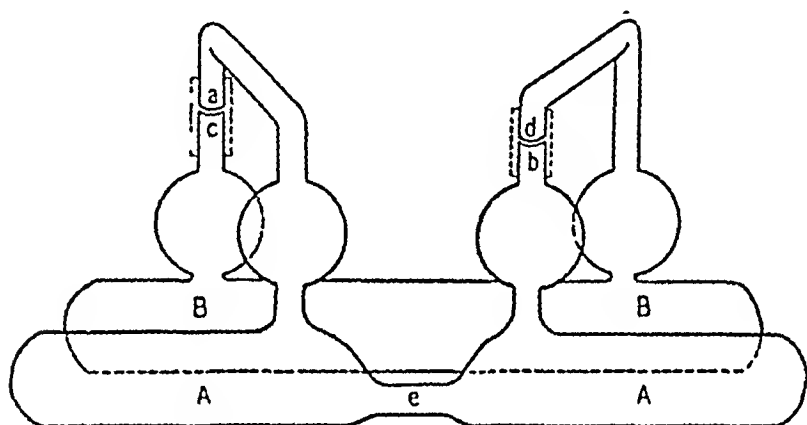
Fig 1

Experimental Technique

After a series of preliminary experiments with various types of apparatus at various temperatures the following apparatus and technique were adopted The apparatus consisted of two glass tubes, A and B, illustrated in Fig 1 Each of these tubes had a capacity of about 60 ml Tube A was charged with egg white and B with yolk After charging, the tubes were connected at ac and bd with heavy walled pressure tubing and the joints tightly wrapped with rubber bands To avoid contact with water they were then mounted and sealed within a 3 liter glass jar which in turn was placed into a 300 liter constant temperature water bath The temperature of the bath was kept at 50° and fluctuated but a few hundredths of a degree The constriction in the centre of the tube A was such that when the tubes were rocked back and forth, egg white would flow from one compartment of A to the other and back again, while the air, with which the yolk and white were in contact, was forced to flow back and forth through the tube B This rocking motion not only kept the liquids in both tubes well stirred but brought the air above them into alternate contact with the liquids in both tubes and thus forced water to distill from the liquid with a lower osmotic pressure to that with a

higher one. Rocking was continued at 50 for about 90 hours in each experiment The same precautions were used in the preparation of yolk and white as previously described (1)

EXPERIMENTAL RESULTS

The data of Table I were obtained with unfertilized eggs

In Experiments 1–4 the osmotic concentrations of yolk and white were left in their natural state In Experiments 5 and 6 sufficient sodium chloride was added to the white to raise its osmotic pressure

TABLE I

No.	Material	Weight of charge	Change in weight
			gm.
1	Yolk	13 319	+0 029
	White	16 360	−0 035
2	Yolk	13 347	+0 030
	White	16 359	−0 040
3	Yolk	12 870	+0 019
	White	16 251	−0 031
4	Yolk	14 632	+0 034
	White	17 985	−0 042
5	Yolk	13 190	−0 038
	White + 0 4 gm NaCl per 100 cc	19 396	+0 028
6	Yolk	13 318	−0 030
	White + 0 4 gm NaCl per 100 cc	18 905	+0 020
7	Yolk	13 409	+0 023
	White + 0 12 gm NaCl per 100 cc	17 911	−0 026
8	Yolk	13 475	+0 014
	White + 0 12 gm NaCl per 100 cc	21 066	−0 026

above that of the yolk by as much as that of the yolk is naturally above that of the white In Experiments 1–4 water distilled from white to yolk while in Experiments 5–6 water distilled from yolk to white In Experiments 7–8 sufficient salt was added to the white to lower its freezing point by one half the difference naturally existing between yolk and white In this case, too, water distilled from white to yolk [1]

[1] That the loss in weight of water on one side is greater than the gain on the other is evidently due to the loss of water from the tubes because of the pressure developed when they were brought to the temperature of the bath A negative pressure was always found to exist within the tubes when, after an experiment, they were brought back to room temperature

Freezing point determinations were made before and after heating yolk and white to 50° for 90 hours and were found not to have been appreciably altered by this period of incubation

DISCUSSION

While these data, because of the nature of these experiments, do not give the exact osmotic pressures of egg yolk and egg white, they do show definitely that the osmotic pressure of egg yolk is greater. No other explanation can be given for the fact that water continued to distill from white to yolk when a considerable amount of salt had been added to the white. It would appear, therefore, that Howard's recent claim (5) that freezing point data previously reported were "erroneously" obtained is unjustified, and that Howard's modification of the writer's freezing point method requires further consideration before it can be accepted

CONCLUSION

It has been shown by a comparison of the relative vapor pressures of egg yolk and egg white before and after the addition of sodium chloride to the white that the osmotic pressure of the yolk is greater than that of the white

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FREEZING POINTS OF ANTI COAGULANT SALT SOLUTIONS

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In connection with the work of Ferguson (1) on the coagulation of blood, it was desirable to know the concentrations of solutions of the commonly used anti-coagulant salts, sodium citrate, oxalate, and fluoride, which possessed the same osmotic pressure as mammalian blood plasma or serum Since freezing point data are given only for the fluoride in the International Critical Tables (2), we undertook to determine the freezing points of aqueous solutions of pure samples of these three salts in the range of physiological interest

Materials

The sodium citrate was a high grade analyzed salt labelled 2 $Na_3C_6H_5O_7$ 11 H_2O, but was found to contain less than the theoretical amount of water It was not possible to attain constant weight on drying either the crystals, or the residue obtained on evaporating a solution at 150° or 180°C. Concentrations were finally determined by evaporating 10 ml samples in porcelain crucibles at 110 and converting the citrate to sodium sulfate by the method used by Foote and Schairer (3) in the determination of sodium fluoride[1] This is an indirect analysis for sodium. Our solutions of sodium citrate had a pH of about 9 0, a value at which the titration curve of Hastings and Van Slyke (4) indicates exact equivalence of sodium and citrate Accordingly this salt was not further purified

The sodium oxalate was an analyzed sample marked "Special, for standardizing according to Sørensen" It lost less than 0 1 per cent in weight on drying at 110 C The purity of the dried samples was confirmed within 1 part in 1000 by titration with a potassium permanganate solution, 0 01442 N which had been standardized against a high grade imported sample of oxalic acid The concentrations of sodium oxalate in the freezing point experiments were determined by titrating 10 ml samples with this potassium permanganate solution

The sodium fluoride, according to the label, contained 0 5 per cent of free acid and 0 2 per cent of sodium fluosilicate. It was purified by neutralizing a saturated

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[1] The authors are grateful to Professor Harry W Foote for his kindness in teaching them the technique of this useful analytical method

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solution to phenol red with carbonate-free sodium hydroxide, and filtering to remove fluosilicate The fluoride was recrystallized by adding an equal volume of alcohol, heating almost to boiling, and cooling to about 2°C The crystals were filtered off, air-dried, ground in a mortar, and dried at 110°C The purified product was tested by conversion to sodium sulfate in platinum dishes according to Foote and Schairer (3) Four analyses agreed within 0 07 per cent, with an average deviation from the mean of 0 02 per cent The average ratio of sulfate found to fluoride taken was 0 18 per cent lower than the theoretical This result might indicate the presence of 0 18 per cent of an impurity volatile under the sulfuric acid treatment, or possibly as much as 0 33 per cent of sodium fluosilicate The concentrations of the solutions in the freezing point experiments were determined by evaporating 20 ml samples and drying to constant weight at 110°C

Sodium chloride of a high grade analyzed brand was used in testing the freezing point technique No analyses were made other than dry weight determinations, but the results of the freezing point measurements showed that the salt was sufficiently pure

Method

The freezing points were determined by a method similar to that of Stadie and Sunderman (5), except that larger amounts of solution were used and the concentrations were determined by analysis or dry weight Temperature was measured by a Heidenhain mercury thermometer with a fixed zero point and scale graduated in hundredths of a degree Centigrade It could be read to the nearest thousandth with a magnifying glass The freezing point vessel was a pint vacuum bottle without a case It was kept in a well stirred, electrically controlled and refrigerated bath containing water and alcohol at −0 50°C ±0 01° The bottle was filled to about one fourth of its capacity with crushed ice made from distilled water This, together with about 50 ml of water or solution, was sufficient to cover the bulb and 2 or 3 cm of the stem of the thermometer The zero point of the thermometer was repeatedly determined with ice and distilled water, and remained constant within 0 001°C The mixture was gently stirred by lifting a glass ring stirrer a few times just before each reading was taken Readings were made at 5 minute intervals, and the temperature was considered constant when three or four such readings agreed to 0 001°C For the determinations with salt solutions, the water was siphoned off and replaced by about 50 ml of solution After thorough mixing of the ice and solution, the bottle was clamped in the bath and a dry pipette for taking the sample was inserted Readings were taken as before until constancy of temperature was attained A sample slightly greater than the amount to be measured was then removed and kept in a closed bottle The mixture in the freezing point vessel was diluted by adding a little distilled water, and after thorough mixing readings were taken as before This procedure was repeated to obtain freezing points of more dilute solutions In some cases the order of the determinations was reversed by adding small amounts of a more

concentrated salt solution to a more dilute mixture   It was possible to check the
zero point with the same ice which had been used for solutions, after washing it
repeatedly in the bottle with cold distilled water   After the samples had reached
20 C , exactly 10 or 20 ml were transferred by standardized pipettes to other ves-
sels for the determination of the concentrations by the methods already described

RESULTS

The results are presented in Fig 1, in which the freezing point
depressions, Δ, in Centigrade degrees, are plotted against the salt
concentrations, C, in moles per liter of solution at 20°C   The points
for each salt, in the range investigated, may be seen to fall close to a
straight line   The lines were drawn on a large scale plot so as to make
the positive and negative deviations nearly equal   The average
deviations of the points from the lines are ± 0.002°C   The equations
of these lines are as follows

$$\begin{aligned}
\text{Sodium citrate} \quad & \Delta = 0.053 + 4.67\ C \\
\text{Sodium oxalate} \quad & \Delta = 0.022 + 4.23\ C \\
\text{Sodium chloride} \quad & \Delta = 0.004 + 3.415\ C \\
\text{Sodium fluoride} \quad & \Delta = 0.002 + 3.39\ C
\end{aligned}$$

In the case of sodium citrate, the concentrations refer to moles of
the tri sodium salt, $Na_3C_6H_5O_7$, and not to the doubled mole some
times used in formulas for the hydrated salt   The open circles on or
near the citrate line represent solutions of sodium citrate in water at
their natural pH value, about 9 0   The half inked circles represent
solutions of sodium citrate brought to pH 7 4 by the addition of very
dilute hydrochloric acid   Within the limits of this method, there is
no difference in freezing point due to the change in pH   The curve of
Hastings and Van Slyke (4) indicates that citric acid is about 99 3
per cent neutralized at pH 7 4

The solutions of the other salts were nearly neutral and had no
appreciable buffer value   No attempt was made to adjust their pH
values

The half inked circles near the sodium chloride line represent our
determinations, and the line was drawn to represent these points
The fully inked circles represent determinations by Scatchard and
Prentiss (6), who used a far more accurate method   We have re
calculated their molal concentrations to moles per liter of solution at

20°   The fact that their points lie only 0 001 or 0 002°C away from our line gives a measure of the reliability of our results
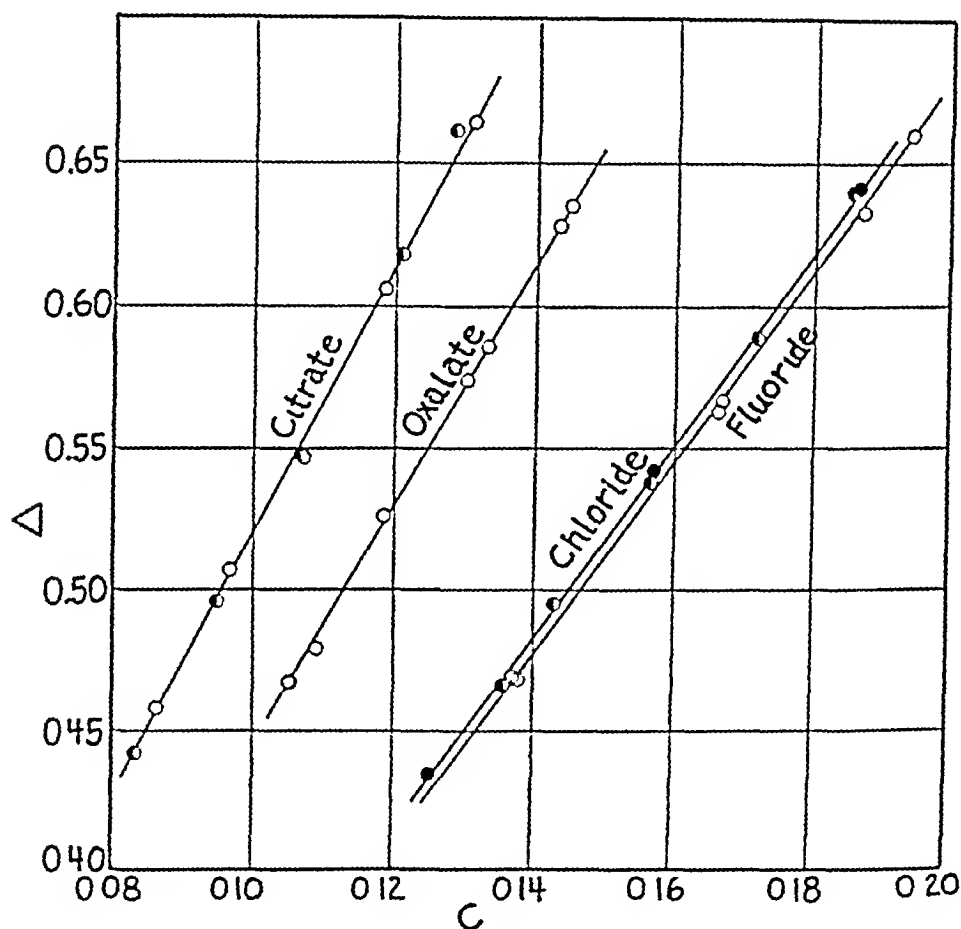
Fig 1 Freezing point depressions of aqueous solutions of sodium salts plotted against concentration

Δ = freezing point depression, °C
C = concentration, moles salt per liter solution at 20°C
O = sodium citrate at pH 9 0 sodium oxalate, sodium fluoride
◖ = sodium citrate at pH 7 4, sodium chloride
● = sodium chloride, from Scatchard and Prentiss

DISCUSSION

The data for sodium fluoride given in the International Critical Tables 2 are based on determinations by Peters (7)   His points for

N/8 and N/6 sodium fluoride solutions fall about 0 025°C above our line Since he used the Beckmann method, and gave no analysis of his salt, we believe our data to be more reliable

Rous and Turner (8) reported that a 3 8 per cent solution of sodium citrate had the same freezing point as a 0 95 per cent sodium chloride solution Leendertz and Gromelski (9) stated that a 1 55 per cent solution of sodium oxalate was isotonic with blood, as was a 3 55 per cent sodium citrate solution ($\Delta = 0\ 56°C$)

For purposes of comparison, the equations given above have been used to calculate the concentrations of the several salts which freeze at $-0\ 56°C$, according to our data The concentrations are as follows

Sodium chloride 0 1628 M, or 0 952 gm per 100 ml
Sodium fluoride, 0 1646 M or 0 691 gm. per 100 ml
Sodium oxalate, 0 1272 M, or 1 704 gm per 100 ml
Sodium citrate, 0 1086 M, or 3 877 gm. of the higher hydrate $Na_1C_1H_1O_7$
$5\frac{1}{2}\ H_2O$, per 100 ml

It is to be emphasized that these concentrations are not given as those of solutions isotonic with average blood samples, but merely as those of solutions freezing at $-0\ 56°C$ Our equations make it possible to prepare solutions having a freezing point identical, within 0 002°C, with that of any biological fluid, provided that the freezing point of the latter is accurately known and falls within the range studied Freezing points of biological fluids may be determined by the method of Stadie and Sunderman (5)

SUMMARY

By a method involving equilibration of ice and solution, and analysis of the solution, freezing point depressions of solutions of sodium citrate, oxalate, and fluoride have been determined over the range $\Delta = 0\ 45$ to $0\ 65°C$ Determinations with sodium chloride solutions have confirmed the accuracy of the method In each case the freezing point depression is given, within 0 002°C, as a linear function of the concentration By the use of these linear equations it is possible to prepare a solution of any of these four salts isotonic with a given biological fluid of known freezing point, provided the latter falls within the range studied

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TEMPERATURE CHARACTERISTICS FOR HEART BEAT FREQUENCY IN MICE

By W J CROZIER G PINCUS, and B RENSHAW

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I

In developing a conception of the probably diverse quantitative relationships between frequencies or speeds of vital processes and temperature in the same or in very similar organisms, studies have been made of the frequencies of breathing movements in inbred strains of mice and in their hybrid offspring (Stier and Pincus, 1928–29, Pincus, 1930–31) The genetic relationships are important as providing a means of testing the definite character of the temperature constants obtained, and are also significant for purposes of genetic analysis (cf Crozier and Pincus, 1928–29, 1929–30 a, b) We undertook to establish for one of the strains of mice used in previous experiments, the Bagg Little inbred albino strain, the relationship between frequency of heart beat and temperature of the organism, as a foundation for further experiments This is the strain of mice also used by Stier (1930, 1933) in experiments concerning "spontaneous activity" and by Pincus, Sterne, and Enzmann (1933) in observations on the development of thermal regulation

The individuals used for the present observations varied in age from 0 5 to 6 days, they were of the 48th to 51st generation of brother by sister mating

II

Over the range of external temperatures 15 to 35° the internal temperature of mice of the ages used in these observations is always above that of the surroundings by an amount varying from 0 1° to more than 2°, the difference in temperature being a function of the internal temperature The results indicate that the difference in temperature

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between mouse and surroundings does not play a significant part in determining the shape of the heart beat frequency curve

The experimental procedure was as follows   A mouse was taped back down upon a wooden substratum in a glass chamber which was mounted upon a brass base   The whole was submerged in a water thermostat   Air was passed through the chamber after being drawn through water bottles and a lengthy coil of copper tubing in the thermostat to moisten it and bring it to the temperature of the water bath   One junction of a copper-constantan thermocouple, carried in a fine glass capillary, was inserted into the rectum of the mouse, the other (reference) junction was placed between brass blocks $\frac{3}{8}$ inch thick at the bottom of the chamber   The thermocouple leads were connected to a circuit involving a Leeds and Northrup Type "R" galvanometer, a differential photocell, and a Leeds and Northrup recording potentiometer [1]   In this way the circuit in the original recording potentiometer was so modified that the original full scale sensitivity (10 inches for 5 mv) was increased so much that 0 5° difference in the temperatures of the junctions of a single copper-constantan couple could be made to record a maximum scale deflection   In the present observations, however, the sensitivity was such that 1 inch deflection was equivalent to 0 473°C   The deflection should be nearly proportional to the temperature difference between the junctions of the thermocouple and this was found true within the experimental error involved in calibrating the thermocouple over the range of temperature difference 0 to 3 5° by means of standard thermometers   It was necessary to place the reference junction between brass blocks serving as a thermal conductor holding the junction at the mean temperature of the tank, without the blocks, cyclic fluctuations corresponding to the heating and cooling of the tank appeared in the curve drawn by the recorder, even though variations in tank temperature were not usually detectable with the standard thermometer immersed in the tank   The thermostat used has been described by Stier and Crozier (1932–33)   The difference between the temperature of the rectum of the mouse and the thermostat tank could be determined with greater precision than the temperature of the tank itself could be measured by a standard thermometer (graduated in 0 10, read to 0 01°)   The internal temperature of the mouse was the sum of the tank temperature and the recorded increment   The normal fluctuations of the mouse rectal temperature probably introduced more uncertainty as to the actual temperature of the mouse

over an interval of time than did errors in either of its two experimentally deter mined components. The heart was assumed to be at the temperature of the rectum Effort was made to determine whether appreciable temperature differ ences occur between various parts of the mouse, notably between the heart and rectum One junction of the thermocouple, encased in a capillary, was inserted into the rectum. The other was placed in n broken-off hypodermic needle which was driven into the region of the heart and withdrawn around the wires leaving the junction in or near the heart A drop of collodion prevented the thermocouple leads from working out of the body of the mouse While it must be understood that the measurements were attended by considerable possibilities for error, the maximum difference found was 0 3°, and the average was only about half of this, the heart temperature being higher Three of the determinations were made at an environmental temperature of about 23 to 24°, one at about 16° The mice were about a week old One determination between the rectum and the brain indicated a small temperature difference It is probable that the temperature difference between heart and rectum (in mice of the ages used) is not over 0 1°, this difference would not affect the subsequent treatment.

The electrical changes accompanying heart beat were led off by platinum wires inserted under the loose skin of the chest and rump to the input of a 4-stage, resistance-coupled amplifier[2] The amplified changes passed to a loud speaker or head phones, and by way of a matching transformer to a string oscillograph (General Radio Company, Type 338-L) By the use of a rotating mirror and screen the deflections of the string could be observed, or by a camera attachment they could be photographed at will The amplitude of the deflections decreased with falling temperature, the lower working limit for detectable electrical dis turbances was at about 15°C

Over the lower portion of the temperature range investigated (15 to 26°C mouse temperature) the film records were frequently supplemented by a number of readings with a stop-watch graduated in 0 01 second Above 26°C , the beat rate became too rapid for accurate counting and film records only were used A procedure frequently employed was to take five sections of film, each lasting several seconds at approximately 30 second intervals over a period of 2 minutes

A timing mechanism (Telechron motor) marked $\frac{1}{3}$ second intervals on the film An integral number of beats were counted. At worst, the position of each beat could be determined on the record to within plus or minus 0 02 second so that, considering a number of beats occupying a length of film equivalent to about 4 seconds, the maximum error in the determination of the average rate over the interval would be at most 1 per cent assuming negligible variation in the 60 cycle A c. supply This is smaller than the variation which was found to occur, even over such short intervals that little change of rate could have been caused by

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slight, normal variations in the internal temperature of the mouse   At any temperature the variation in rate over a period of several minutes, though smaller than found in many cases for rate of heart beat and other vital processes in various organisms, amounted to about 5 per cent of the mean   The range of variation at any temperature was roughly a constant fraction of the mean, although not enough data were taken at each temperature to warrant statistical analysis (*cf* Crozier, 1929)

The young mice were generally active at the end of an experimental run of 12 to 18 hours' duration   Only a very few died during the course of the observations, and these deaths may have been caused by the piercing of the rectum by the capillary containing the thermocouple junction   In the few cases where a "hysteresis effect" was observed in the frequency of heart beat, the change was more often an increase than a decrease

No clear-cut change was observed in frequency of heart beat when the mouse became active in a cycle of "spontaneous movements."

At any constant temperature the frequency of heart beat increases with weight (age) of the mouse   The relationship between frequency of heart beat and weight is approximately parabolic   There is no change, however, in relation of frequency to temperature within the range of weights involved

As we have found in a number of other cases (*cf* Pincus, 1930–31), the frequency at the first temperature to which the mouse is adjusted is generally far off the smooth curve connecting the points taken subsequently   This is true even when adequate time is allowed for thermal equilibration   Generally the frequency is too low, and it shows a drift for several hours, if the temperature be changed during this time, the frequency of heart beat at the second or any following temperature to which the animal is subjected does not exhibit this drift

The precise relationship between frequency and temperature is independent of the order of temperature changes, as was demonstrated by the taking of one or more check points in each experiment

III

Data from fourteen sets of observations with different young mice of the inbred albino strain are summarized in Fig 1   In this figure it
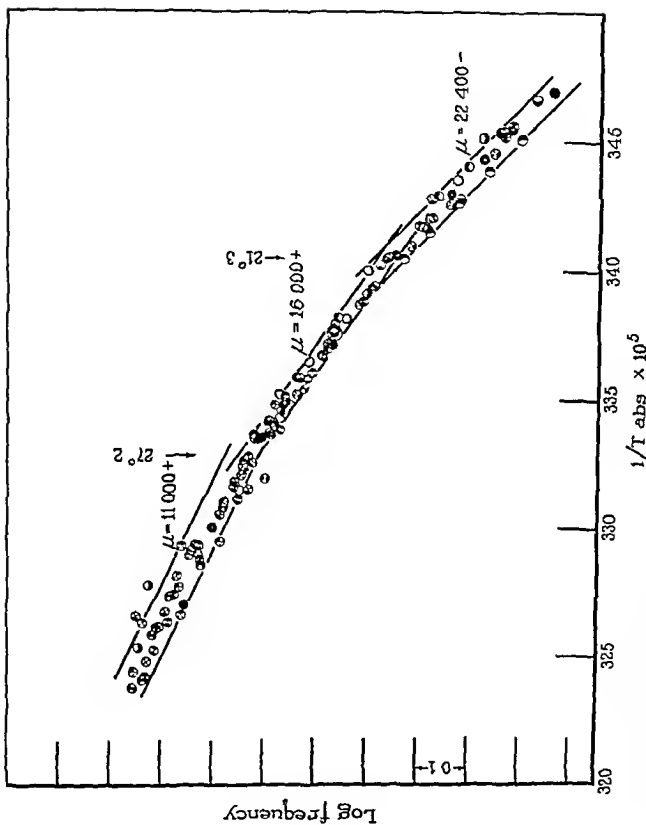
Fig 1 Mass plot of the data from fourteen individuals of the inbred albino strain The various symbols denote single individuals See text.

is intended to compare the *slopes* of the lines drawn through the observations in terms of the Arrhenius equation,

$$\ln frequency = -\frac{\mu}{RT} + \text{constant}$$

The several sets of observations have therefore been brought together by considering that the data in each series may be multiplied by an arbitrary constant so adjusted that the respective curves coincide at 25° There are apparent two critical temperatures, where the slope constant μ changes rather abruptly, one of these is located at slightly higher than 20°, the other at 27°

The values of μ (Fig 1) are 22,400− from 15 to 20°+, 16 000+ from 20+ to 27°, and 11,000± from 27 to 35°+ The apparent greater scatter of the observations over the upper and lower temperature ranges is partly, at least spurious, since the individual curves were brought together in the middle, and there is some difference in the exact location of the critical temperatures The breaks in the curves occur at temperatures which have frequently been found as critical (cf Crozier 1925-26 a b) It is interesting to note that for this same strain of mice 20° − was found to be a critical temperature for frequency of respiratory movements, the values of μ for respiratory movements were however, 14 000± calories above 20° and 34,000± (or 14 000±) calories below 20° (Pincus, 1930-31) This indicates that in these mice the organism does not determine a uniform temperature characteristic for all processes (see also Stier and Wolf, 1932-33)

These experiments indicate that the Arrhenius equation holds for the temperature relations of heart beat frequency in intact mammalian hearts as has already been shown for isolated hearts (Crozier, 1925 26 b It is rather remarkable that over any given temperature range the value of μ is the same from mouse to mouse The fact that we are dealing with a highly inbred strain of mice indicates a possible genetic basis for this uniformity It is hoped that the proper experiments with other strains of mice will elucidate this point

heart beat frequency is related to body temperature the values of the temperature characteristic for the inbred albino strain used are 22,400— calories over the range 15 to 20°+, 16,000± calories from 20+ to 27°, and 11,000± calories from 27 to 35°+

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NATURE OF THE ACTION CURRENT IN NITELLA

III Some Additional Features

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This paper describes certain forms of the action current which might be explained by assuming that the outer protoplasmic surface shows no rapid electrical change

The records (made as previously described[1]) are in all cases mono phasic The cells were arranged as shown in Figs 1 and 2 When the common contact, F, was not killed, simultaneous records of C, D, and E showed that the common contact was quiescent and made no contribution to the recorded changes in P D No electrical or mechanical stimulus was applied, though in some cases the solutions used may have acted as chemical stimuli (care was taken to avoid any disturbance due to evaporation)

Let us now consider some of the records The upward movement in Fig 3 is due to a partial loss of the P D across the protoplasm This appears to consist of two separate[2] P D 's, one at Y and another at X (Fig 4) We suppose that in the usual form of negative variation both of these disappear when the action current flows outward This produces a rapid electrical change in X (and makes a second peak in the action curve) as K^+ sweeps across the protoplasm[4] and out through X to the cellulose wall in which its lengthwise flow chiefly occurs (Fig 5a)

When the non aqueous layer W offers less resistance (owing to its

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[1] Hill S E and Osterhout, W J V *J Gen Physiol* 1934–35, 18, 377

[2] Except in Fig 14 these records were continuous as shown in previous papers (Osterhout, W J V, and Hill, S E, *J Gen Physiol*, 1930–31 14, 473, 611) In order to save space the record of only one of these spots is given in each case.

[3] The P D s at Y and at X appear to depend chiefly on the difference in the concentration of K^+ on their opposite sides In addition organic substances in W may play a part

[4] *Cf* Osterhout, W J V, *J Gen Physiol*, 1934–35, 18, 215

thickness or content of electrolyte) or when[5] X or the cell wall is more resistant so that the lengthwise flow of the outgoing current occurs chiefly in H^- (Fig 5 b) instead of in the cellulose wall, no rapid electrical
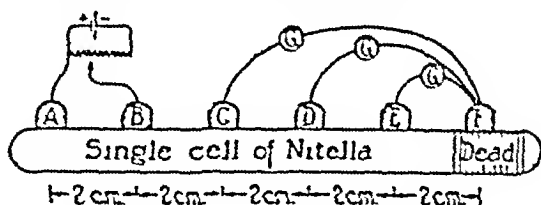
Fig 1 Arrangement for testing *Nitella* cells   *GGG* represent string galvanometers (three strings inserted in the single magnetic field of a Type A Cambridge string galvanometer) with vacuum tube amplifiers, arranged as short period voltmeters   Absorbent cotton, moistened with the contact solution, connects the cells to saturated calomel electrodes
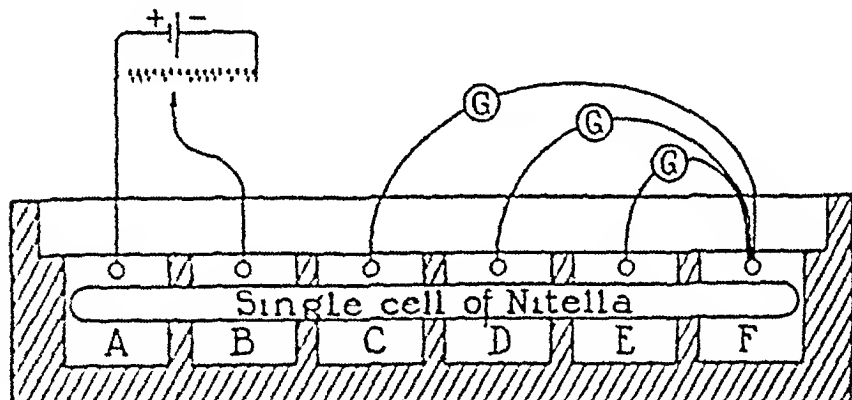
Under these conditions we should expect only one peak in the action curve because too little K^+ would reach V to make a second peak   As the outward movement of K^+ would be relatively small its backward movement might be correspondingly quick so that recovery would be rapid   Both of these expectations are realized as shown in Fig 3
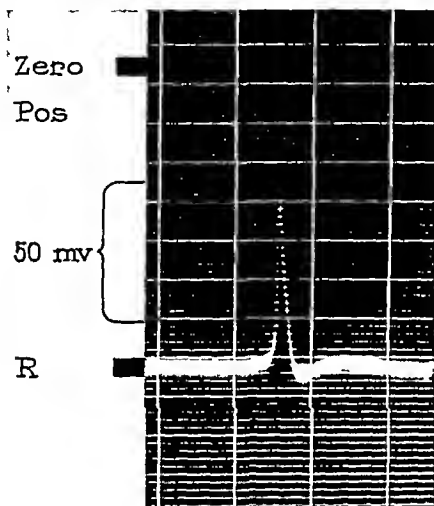
FIG 3 Photographic record of experiment arranged as in Fig 1 employing contacts C and F only F was killed with $CHCl_3$ before the record started to give monophasic response C was in contact with 0 001 M KCl The cell had been kept for 9 days in tap water   The vertical lines are 5 seconds apart   Tempera ture about 21°C R represents the complete resting state

The action curve does not go to zero   it has a single peak and rapid recovery This would be expected if the curve were due to loss of P D at I (Fig 4) leaving intact the P D at V   There is some after positivity

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to carry it across II   Hence the lengthwise current would tend to flow more in the inner than in the outer portion of II

[7] This would also be true if K reached V and its mobility in V were low   but in that case the resistance of V would be correspondingly high (cf footnote 4)

In this case the curve does not go to zero   This is to be expected if the protoplasm is in the condition shown in Fig 4, for the upward movement will cease as soon as the P D across Y disappears after which there remains[8] the P D of X As the latter is presumably variable the behavior of the action curve would be expected to correspond and this
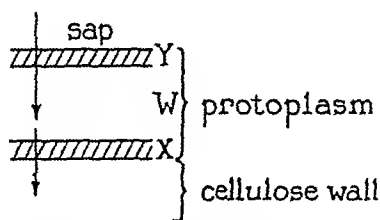
Fig 4   Hypothetical diagram to show the P D in the protoplasm   The arrows show the direction in which the positive current tends to flow   It will be noted that lengthening either arrow would cause the action curve to move downward, i e , in the direction of the arrow   The length of the arrow is supposed to depend largely on the concentration gradient of K^+ across the layer
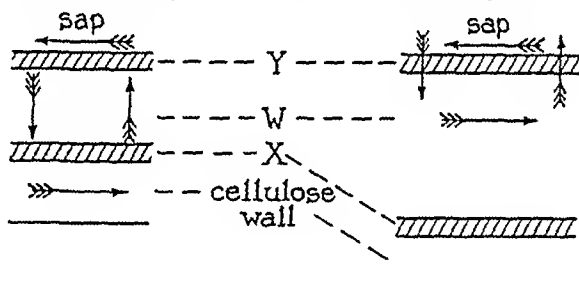
Fig 5 a   Fig 5 b

Fig 5 a   Hypothetical diagram to show the flow of the action current under ordinary conditions

Fig 5 b   Hypothetical diagram to show the course of the action current when its outgoing lengthwise flow is largely confined to the protoplasm   This may be due to greater thickness or greater electrolyte content of W or to increased resistance of X or of the cellulose wall

expectation is fully borne out by observation as the amount by which the curve falls short of zero is quite variable   Occasionally a single peaked curve reaches zero in which case we suppose that there is practically no P D across X This is especially common in *Chara* [9]

[8] The P D of X apparently depends largely on the concentration of K^+ in W as compared with that in the external solution, but it may depend also on organic substances in W Cf Osterhout, W J V , *Ergebn Physiol* , 1933, 35, 1014

[9] In *Chara* (cf footnote 4) the mobility of K^+ in X appears to be no greater

It may be added that, as previously explained,[4] the loss of P D appears to depend in part on an increase of permeability in Y and if this took the form of an actual breakdown (partial or complete) of Y it might produce a loss of P D even if little or no movement of K^+ took place but such a breakdown would undoubtedly facilitate the outward movement of potassium across Y

Let us now consider a phenomenon which is observed occasionally in connection with normal action currents but is more common with
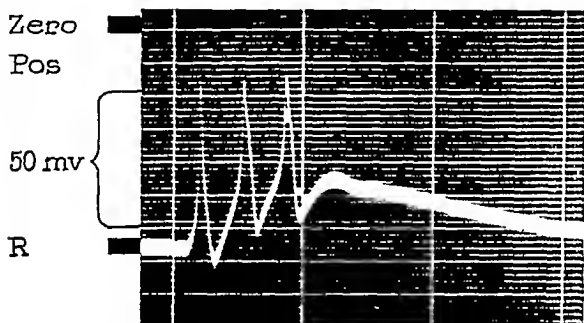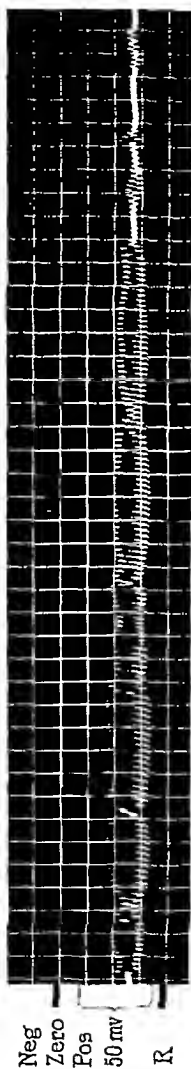

FIG 6 Photographic record of an experiment arranged as in Fig 1 employing contacts C and F only F was killed with $CHCl_3$ before the experiment started to give monophasic recording at C C was in contact with Solution A (*cf* Osterhout W J V and Hill S E *J Gen Physiol* 1933–34, 17, 87) in which the cell had been kept for 10 days The vertical lines are 5 seconds apart Temperature about 20 R represents the complete resting state

For explanation see Fig 7

the form of action current just discussed This is the production of a series of action currents without complete recovery An example is seen[10] in Fig 6

than that of Na^+ hence the resistance of X is correspondingly great and the X arrow is probably small

[11] The suspicion that any part of any of the curves shown in this paper may be instrumental in origin can be ruled out completely When instrumental disturbances occur they are entirely different in character being much more rapid less regular and of smaller amplitude

In Fig 6 the first upward movement is presumably produced by K^+ moving from the sap to the outer surface of I^- thereby reducing the P D of I^- to zero and causing the I^- arrow to disappear   The X arrow is supposed to suffer little alteration because little K^+ is carried across II to X and the curve does not go to zero because after the I^- arrow has disappeared the X arrow remains and the distance between zero and the curve at the end of any upward movement measures the
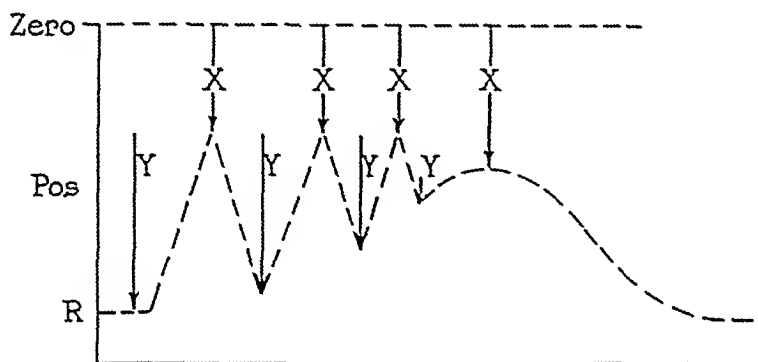
FIG 7 Hypothetical diagram to show the P D across X (X arrow) and across Y (Y arrow) these P D 's are presumably due to the potassium gradient across X and Y We suppose that the X and Y arrows may vary independently because K^+ need not be uniformly distributed across II

When the first Y arrow has disappeared we suppose that the first upward movement has come to a stop this applies in all cases, including the last arrow which is very short in agreement with this the last upward movement is very small The Y arrow is in all cases regarded as equal to the next upward movement of the curve   The last X arrow is longer than those which precede it presumably because the K^+ gradient across X has increased (the Y arrow is shorter because the K^+ gradient across Y has decreased)

The complete resting state is denoted by R (after the second and third upward movements the curve goes to an incomplete resting state)

length of the X arrow at that time   The length of the Y arrow is given by the extent of the upward movement (assuming that the Y arrow disappears entirely as is most probable[4])

After the second action current in Fig 6 recovery is incomplete [11]

[11] This recalls the tetanization of muscle and nerve   The incomplete resting state can persist in *Nitella* after action currents have ceased as shown in Figs 9 12 and 13   It should be remembered that in these cases no intentional stimulus was applied and under the conditions of the experiment loss of water at any point could not be responsible for the result

We interpret this to mean that not all of the K^+ which moved into W has returned to the sap and the Y arrow becomes shorter (as shown in Fig 7) because the K^+ gradient across Y becomes less as the excess K^+ remaining in W has not yet reached X the X arrow remains unchanged, but a little later some K^+ reaches X and in consequence the X arrow lengthens
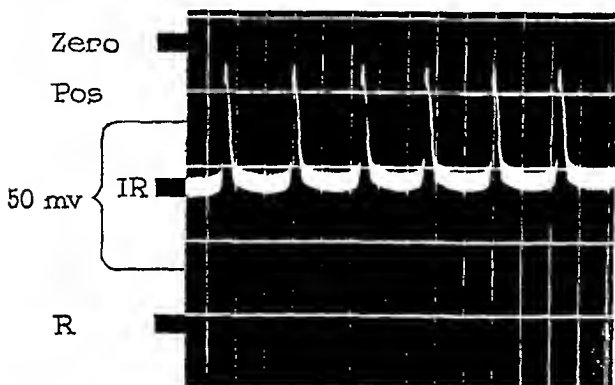
Fig 8 Photographic record of an experiment arranged as in Fig 1 employing contacts C and F only F was not killed but did not change during the recording C was in contact with 0 01 M NH_4Cl (pH about 6 0)   The cell had been kept for 2 days in Solution A   The vertical lines are 5 seconds apart   Temperature about 21°C R represents the complete resting state and IR the incomplete resting state

The first action current in this series (not shown here) carried the curve upward from the resting state (R) and the figure shows a series of action curves returning each time to an incomplete resting state (IR)   This might be interpreted as meaning that approximately the same amount of K^+ moves into the sap each time during recovery but this amount is not sufficient to cause complete recovery The P D across X (X arrow see Fig 7) remains constant presumably because no K^+ moves from the sap to X

Complete recovery restores the protoplasm to the normal resting state which is designated by R the incomplete resting state produced by incomplete recovery may be called IR

When recovery is incomplete we suppose that the K^+ moving into

W does not all return to the sap and it is evident that with successive action currents we may have

1   The same amount is returned each time so that the degree of recovery remains the same for all the action currents   An example is shown in Fig 8   Here the first action current (not shown in the
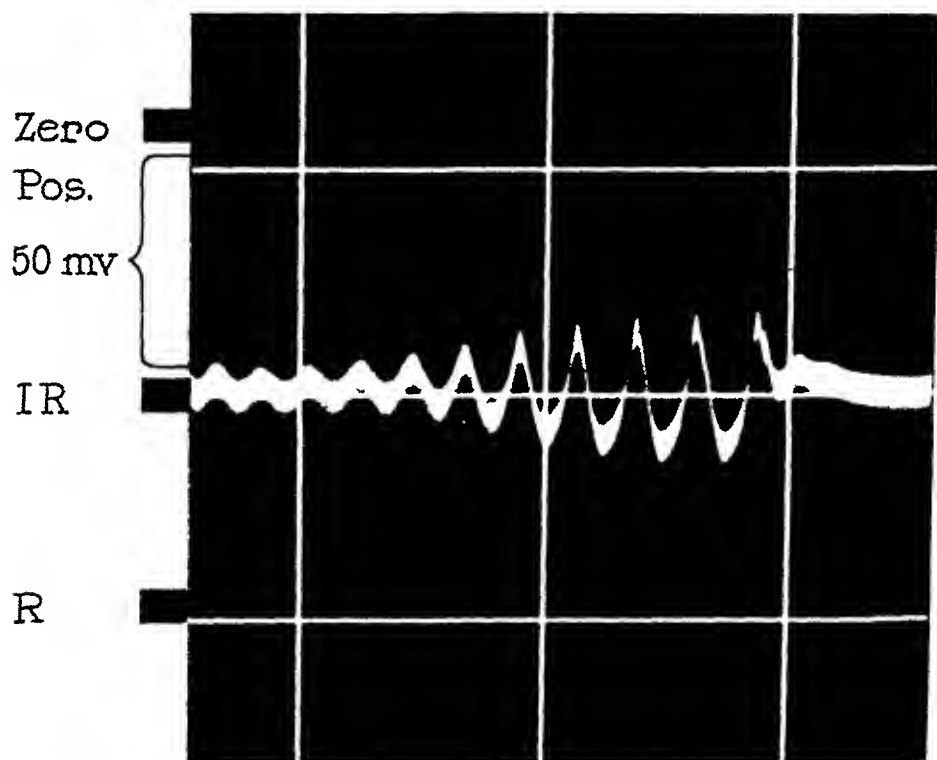
FIG 9 Photographic record of an experiment arranged as in Fig 2, employing contacts *D*, *E*, and *F* only   *F* was not killed but did not change during the recording   all spots in contact with 0 01 M NH₄Cl (about pH 6)   The cells were kept for 5 days in Solution A   The vertical lines are 5 seconds apart   Temperature about 20°C

This record might be interpreted as meaning that a little more K^+ is returned to the sap with each successive recovery thus increasing the P D across *Y* (*Y* arrow, Fig 7) and shortening the P D across *X* (*X* arrow)

record) carried the curve up from the normal resting state (*R* in the figure) and this was followed by a series of action currents   Recovery was incomplete but its extent was quite uniform

2   A little more K^+ is returned to the sap with each successive action

current so that the degree of recovery increases as time goes on   Such a case is seen in Fig 9   Here the decrease of K^+ in W not only length
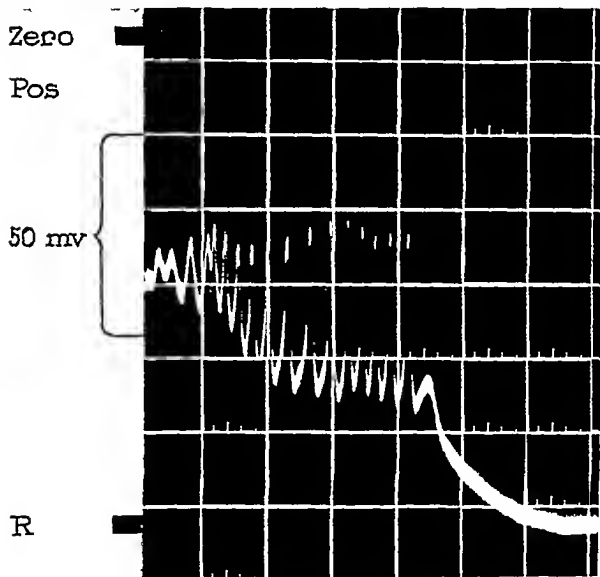
FIG 10 Photographic record of an experiment arranged as in Fig 2 employing contacts C and Γ only F was killed with $CHCl_3$ before the record started to secure monophasic recording at C C was in contact with 0 01 M NH_4 acetate (pH about 6)   The cells had been kept for 29 days in Solution A.   The vertical lines are 5 seconds apart   Temperature about 22 C   The initial action curve (not shown here) was due to electrical stimulation at B (100 mv D C)

This record might be interpreted as in Fig 9 except that the P.D across λ (λ arrow) does not shorten presumably because only the inner part of W loses K^+

ens the Y arrow but it also shortens the λ arrow   In Fig 10 this shortening of the λ arrow does not occur, presumably because only the inner part of W loses K^+
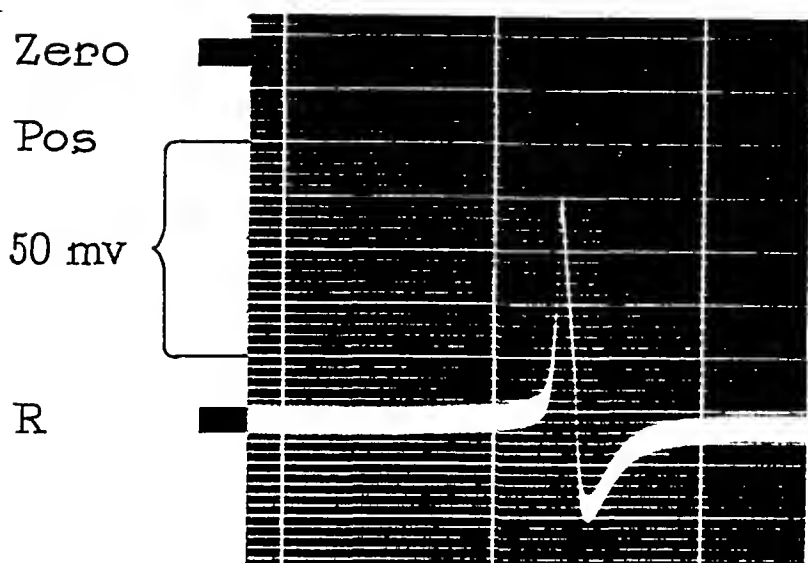
FIG 11 Photographic record of experiment arranged as in Fig 1, employing contacts C and F only F was killed with $CHCl_3$ before the record started, to secure monophasic recording at C C was in contact with Solution A, in which the cell had been kept for 10 days   The vertical lines are 5 seconds apart   Temperature about 20°C R represents the complete resting state

This record shows after-positivity (for discussion see text)
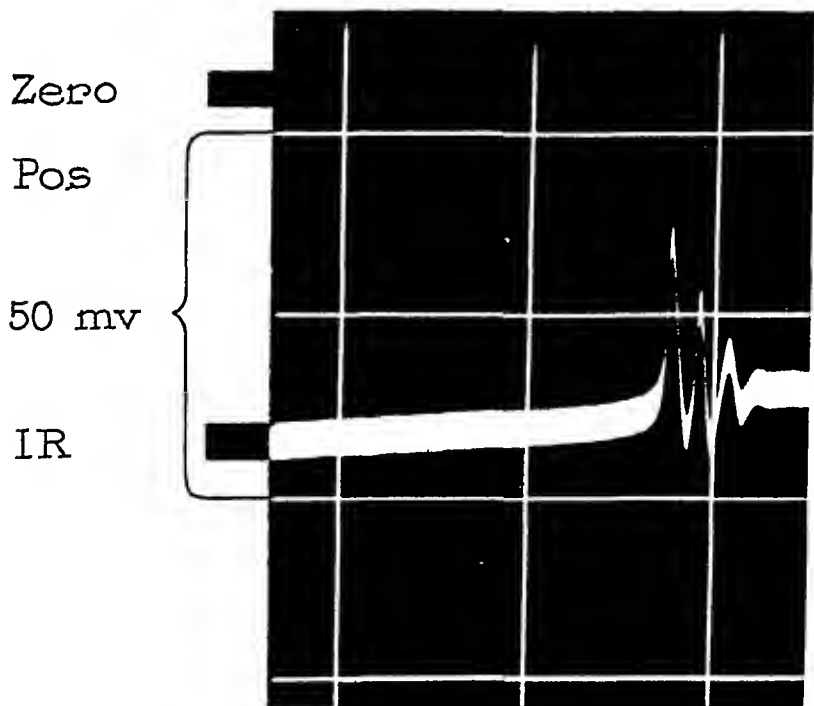
FIG 12 Photographic record of an experiment arranged as in Fig 2, employing contacts C, D, and F F was not killed but it did not change during the recording C (the only spot whose record is shown here) was in contact with 0 01 M NH_4Cl (pH about 6) as were also D and F The cell had been kept 5 days in Solution A Temperature about 20°C   The vertical lines are 5 seconds apart

This record shows a shortening of the Y arrow and lengthening of the X arrow which might be due to increase of K^+ in II

FIG. 13 Photographic record of an experiment arranged as in Fig. 1, employing contacts C and F only. F was killed with CHCl₃ before the record started, to give monophasic recording at C. C was in contact with 0.01 M NH₄ acetate, pH 7.0, in which the cell had been soaked for 24 minutes before the record started, previous to this the cell had been kept in Solution A. The vertical lines are 5 seconds apart. Temperature about 22°C. R represents the complete resting state.

The record shows several cases where the p.d. across Y (V arrow) increases (as shown by the increasing length of the upward excursion) as the p.d. across X (Λ arrow) decreases (followed by the reverse relation). This would be expected if the result depended on the decrease of K in II' followed by an increase.

Neg.
Zero
Pos.
50 mv.

R

FIG 14

510
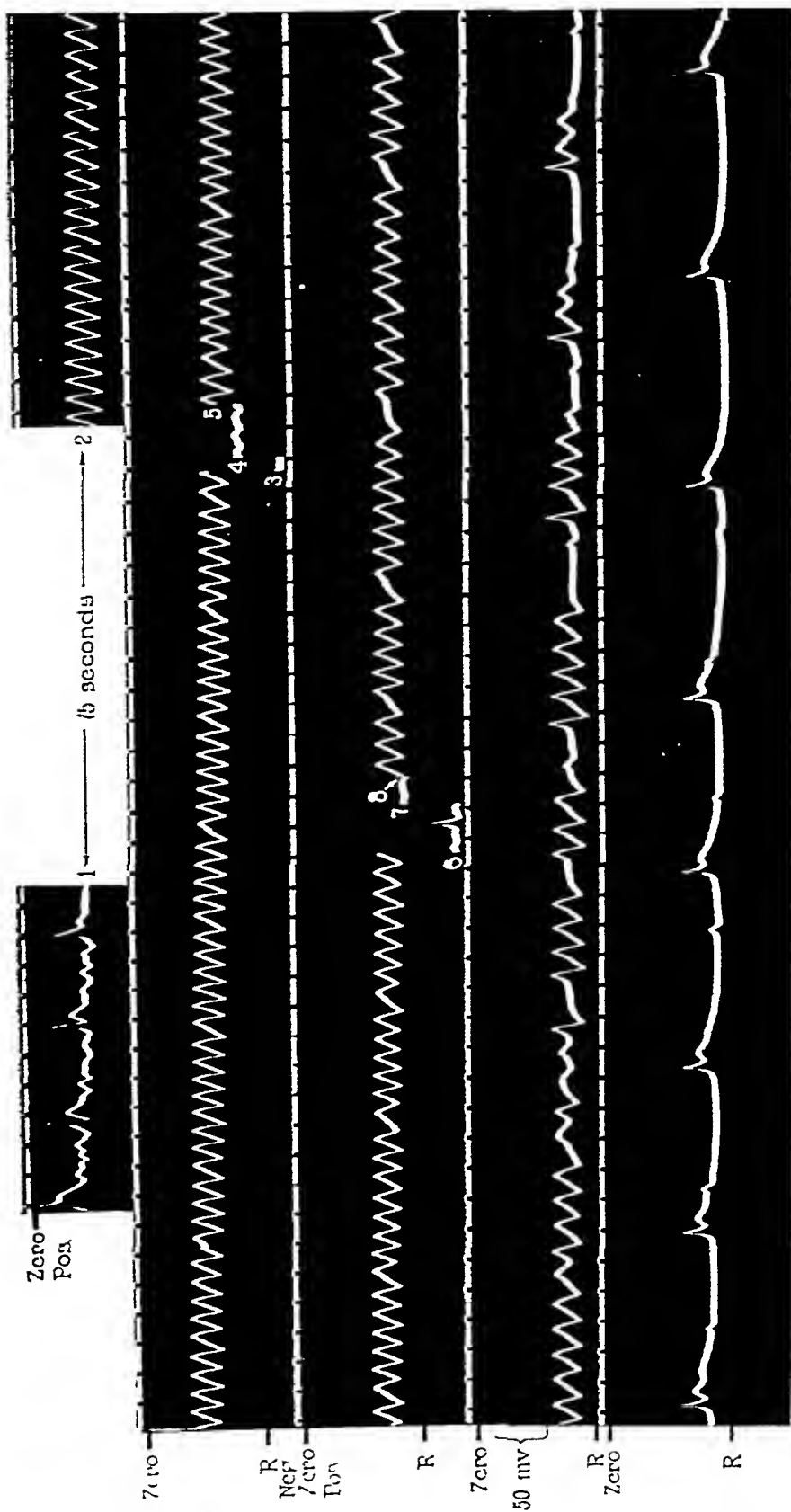
It seems possible that the inward movement of K^+ may sometimes raise K^+ in the sap above the normal and so produce the after positivity seen in Fig 11 This might be brought about in other ways as suggested elsewhere [4] It is of comparatively rare occurrence

3 A little less K^+ is returned to the sap with each successive action current so that the Y arrow becomes less and less We see this in Fig 6 which also shows that as time goes on some of the K^+ reaches X and lengthens the X arrow The same thing is shown more strikingly in Fig 12 here one might get the impression that the P D found at the start (IR) is restored by the lengthening of the X arrow instead of by the lengthening of the Y arrow If such a procedure restored the true resting state (R) it might be termed false recovery to distinguish it from true recovery in which the P D is restored by the lengthening of the Y arrow After false recovery action currents could be produced by movements of K^+ across X Evidently false recovery is theoretically possible but it does not seem probable that it plays an important rôle

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FIG 14 This photographic record was continuous (except between the places marked 1 and 2) for purposes of reproduction it was cut into strips which are arranged consecutively The cell was arranged as in Fig 1 Only one string was used which was connected to D except as otherwise noted (F was killed with chloroform before the record started) C was in contact with 0 001 M $MgCl_2$ D with 0 01 M $MgCl_2$ F with 0 1 M $MgCl_2$ At 3 the string was switched to E at 4 to C at 5 back to D at 6 to F at 7 to C at 8 back to D

At the start D is 55 mv positive to F and the first action current reduces this to zero this action curve has a double peak The subsequent action curves do not go to zero and after a time only one peak is observed But at the end of the record a double peak again appears Otherwise the behavior resembles that in Fig 8

From the behavior of the string when switched to C and E we may infer that these spots were not continuously behaving like D

Temperature about 20 C Previous to the record the cell had been kept for 15 days in Solution A The time marks (at the upper margin) are 5 seconds apart R denotes the complete resting state

The regularity of the oscillations in some parts of the record might raise the suspicion that they were due to instrumental causes but this has been excluded by a careful study of all the possible instrumental disturbances It will also be noticed that toward the end these oscillations pass over into action currents which are more nearly normal

Zero
Pos
R

Net
Zero
Pos
R

Zero

R

Zero

100 mv {

Zero

R

Zero

R

Zero

R

Zero

R

Zero

R

512
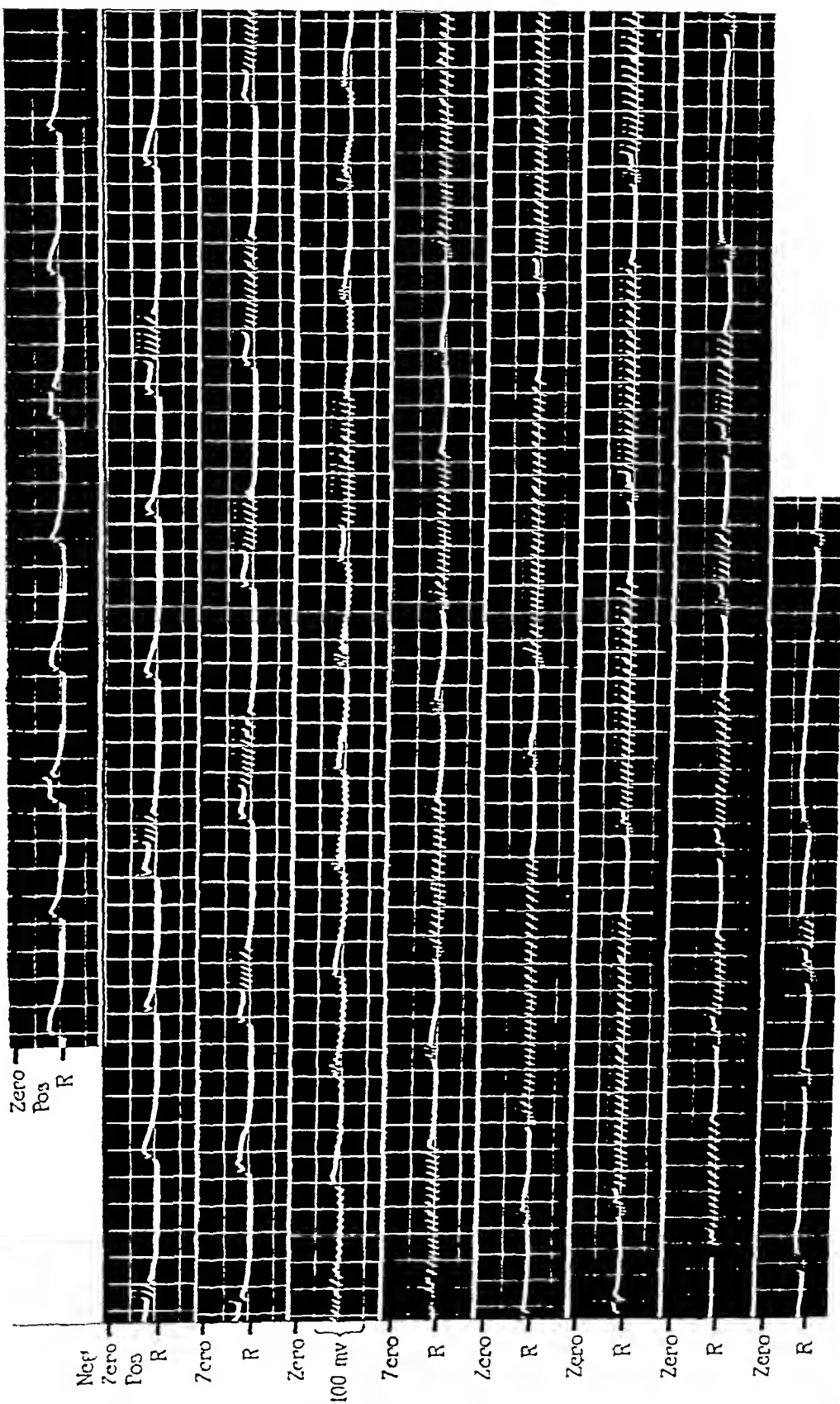
4 In such cases as those shown in Fig 13 we have the situation described under (2) followed by that described under (3), i e, a decrease of K^+ in W followed by an increase

These considerations would lead us to expect that as K increased in W the Y arrow would shorten and in proportion as K^+ reached X the X arrow would lengthen also that if K decreased in W the X arrow would shorten (or in some cases remain constant[12]) and the Y arrow would lengthen But we should not expect both X and Y arrows to lengthen or shorten simultaneously These expectations are fully borne out by observation

Figs 14 and 15 show interesting cases of a transition from a double peaked to a single peaked action current One way of accounting for this would be to assume that some of the K^+ and Na^+ which moves into W is not returned during the process of recovery[13] and that in consequence W becomes more conductive so that the action current becomes single peaked An increase of organic ions in W might have the same effect The opposite process would change a single to a double peak as seen in the last part of the record shown in Fig 14

The action curves in these two figures can be interpreted in the manner already indicated They recall some of the records obtained by Adrian[14] and by Hoagland[15] with nerve

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FIG 15 This photographic record was continuous and for purposes of reproduction was cut into strips which are arranged consecutively The cell was arranged as in Fig 2 only E is shown C and D were recorded but are not shown here they prove that the record was monophasic since there was no activity at F (F was not killed) C was in contact with $0\,01$ м NH_4Cl (pH about 6), D with $0\,01$ м $CsCl$, E with $0\,01$ м $NaCl$.

The first action curves are double peaked (the curves do not go to zero), but after a time single peaks appear, then double peaks recur and are followed by single peaks, this happens several times. Later on bursts of single peaks occur which recall some of the records obtained by Adrian and by Hoagland with nerve.

Temperature about 22°C Previous to the record the cells had been kept for 15 days in Solution A Time marks are 5 seconds apart

This record also shows several of the features discussed earlier in the paper

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[1] It would remain constant if the concentration of K diminished in the inner part of W but not in the outer part

[13] This may or may not lengthen the X arrow depending on how much K^+ reaches X

[14] Adrian, E D , The basis of sensation, London, Christophers 1928

[15] Hoagland, H , *J Gen Physiol* , 1932–33, 16, 695, 715

In conclusion it may be desirable to state that the forms of the action curve here described are relatively infrequent and constitute less than 5 per cent of the curves observed by us   The first cases of this sort were observed in 1925 in experiments carried out by E S Harris in collaboration with the senior author and additional ones have been noted from time to time up to the present

When three places on the cell were recorded it was found that in some cases the action current was confined to one spot but in other cases it was propagated to the other spots where it was sometimes quite similar but in other cases varied in form and amplitude

The hypothesis outlined in this paper seems to involve nothing improbable and may be useful in bringing under a single point of view numerous otherwise puzzling forms of the action curve

SUMMARY

Several forms of the action curve are described which might be accounted for on the ground that the outer protoplasmic surface shows no rapid electrical change   This may be due to the fact that the longitudinal flow of the outgoing current of action is in the protoplasm instead of in the cellulose wall   Hence the action curve has a short period with a single peak which does not reach zero

On this basis we can estimate the P D across the inner and outer protoplasmic surfaces separately   These P D 's can vary independently

In many cases there are successive action currents with incomplete recovery (with an increase or decrease or no change of magnitude)

Some of the records resemble those obtained with nerve (including bursts of action currents and after-positivity)

ELECTRICAL FACTORS INFLUENCING THE RATE OF FILTRATION OF AQUEOUS ELECTROLYTE SOLUTIONS THROUGH CELLOPHANE MEMBRANES*

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Various observers have reported changes in filtration rate of aqueous electrolyte solutions through membranes with changes in concentration, independent of viscosity (Brukner, 1926, Manegold and Hofmann, 1930 a, b, 1931, Duclaux and Errera, 1924, 1926) In general the addition of an electrolyte increased the rate of filtration above that of water Erbe (1932) failed to find any effect of KCl on membrane permeability

Two types of explanation of the increased impermeability in the presence of electrolytes have been offered The first is that of Duclaux and Errera, that the lower rates with water and very dilute solutions are due to an electroosmotic back transport with stream potential as the driving voltage, which is diminished with increase in conductivity This same concept has been applied to flow through capillary tubes (Abramson, 1931, Bull, 1932, see, however, Reichardt, 1933 for a discussion of the inadequacy of Bull's application of this concept to the effect of electroosmotic back transport on stream potentials) and, with diffusion potentials substituted for stream potentials, to abnormal osmosis (Bartell, 1914, 1923, Freundlich, 1916, Loeb, 1922, Sollner, 1930, Grollman and Sollner, 1932)

The second is that of Brukner and Manegold and Hofmann, that the changes in permeability are due to changes in the thickness of a layer of oriented water dipoles held on the pore walls by electrical forces For evidence that adsorbed water layers exist see Kolkmeijer

and Favejee, 1933, Liepatoff, 1926, Nutting, 1927, Boswell and Dilworth, 1925, Dumanksi, 1933, and for evidence that the development of such layers is a function of the electrokinetic potential see Buzagh, 1930, Zocher and Jacobsohn, 1929, Pauli, 1929

It occurred to us that the question of the influence of electrical factors on filtration rates could best be studied by comparing the filtration rate of a solution in which these factors are absent, *i e* at the isoelectric point, with those of solutions where they are present Since both effects discussed above decrease permeability, a maximum filtration rate at the isoelectric condition is predicted Our experimental findings confirm this prediction

Determination of Filtration Rates —The cellophane "600" membranes were soaked in the solution to be filtered for 24 hours previous to the filtration The membrane (diameter of filtering surface 7 0 cm) was put into a porcelain filtering apparatus of the type illustrated by Jander and Zakowski (1929) The membrane was supported on each side by a perforated porcelain disc in direct contact A large rubber stopper was sealed into the top by beeswax-rosin cement, a vertical glass tube was tightly fitted into a hole in this stopper Pressure applied to the compartment below the membrane was read with a mercury or water manometer and the filtration rate determined by reading with a horizontally mounted microscope fitted with a micrometer ocular the rate of rise of the meniscus in the tube The whole apparatus was immersed in an electrically controlled water bath at 25° $\pm$ 0 01 The pressure (25 mm Hg) was applied when the apparatus was first put into the bath and readings begun after 2 hours With thorium solutions of concentration M/50 or less, any further change in rate with time was so slow as not to exceed, within the period of observation (approximately a half hour), the limits of experimental error With more concentrated thorium solutions (M/10), the filtration rate slowly decreased over a period of several hours This falling off with time is undoubtedly due to a mechanical blocking of the membrane, caused by the adsorption of colloidal thorium hydroxide onto the pore walls Repeated determinations on a given membrane showed a maximum fluctuation of $\pm$1 5 per cent and an average of $\pm$1 per cent A given membrane was used for a series in the various solutions The rate with water remains constant after repeated determinations The absolute values for the filtration rates with water on three membranes are 1 51, 1 48, and 1 09 $\times$ 10^{-8} cc /sec /cm ^{2}/cm H_2O The first two figures are on membranes cut from the same piece of new cellophane, the last was on a sample cut from the same piece a few months later Whether this decrease in permeability with age of the dry and unused membrane is a consistent occurrence we cannot say These values may be compared with the figure of 5 6 $\times$ 10^{-9} (McBain and Kistler (1928), Table I) on cellophane 600, and with

the figure of 5.5×10^{-9} (Manegold and Viets (1931), Table I) for a sample of "*Einmachecellophan*" which was 0.006 cm. thick, McBain and Kistler found cellophane 600 to be 0.0098 cm thick.

The procedure followed has been to determine on a given membrane the filtration rate of water then of the various thorium chloride solutions and finally of water. The water rates at the beginning and end of a series were the same. This has been carried through for three membranes the results are shown in Fig 1. The percentage deviation in filtration rate from that of water (indicated by the horizontal line) is plotted against the negative logarithm of the molar concentration of ThCl₄. The rate is seen to reach a maximum at a concentration of $4 \times$
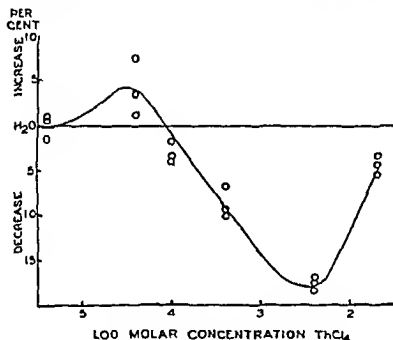
FIG 1 Filtration rate through cellophane as a function of concentration, ThCl₄, O

10^{-5} M and then to fall off, becoming from 15 to 20 per cent less than the water rate in a concentration of 4×10^{-3} M ThCl₄. Further increase in the thorium concentration results in an increase in filtration rate.[1]

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[1] This fact, that in 2×10^{-2} M ThCl₄ the filtration rate is considerably faster than in 4×10^{-3} M ThCl₄ demonstrates conclusively that the changes in filtration rate with varying thorium concentration, up to a concentration of 2×10^{-2} M are not due to mechanical blocking of the membrane by colloidal thorium hydroxide. If this blocking effect were an important factor, the filtration rate with 2×10^{-2} M would be less than with 4×10^{-3} M. As has been stated previously however, in solutions more concentrated than 2×10^{-2} M ThCl₄ a sufficient amount of the colloidal material is present to cause a noticeable blocking, hence filtration values for more concentrated solutions cannot be obtained

Correlation of Changes in Filtration Rate with Zeta Potential —The zeta potential of cellophane as a function of thorium concentration is discussed in the accompanying paper of this series   It was demonstrated that, while the absolute magnitude of the zeta potential cannot be determined on cellophane membranes, nevertheless the isoelectric point could be accurately located at a concentration of approximately 4×10^{-5} M $ThCl_4$ Also, the curve of electroosmotic velocity as a function of thorium concentration obtained with cellophane membranes (see Fig 1 of the accompanying paper) indicates that the zeta potential of reversed sign obtained in 4×10^{-3} thorium chloride is much greater than the potential in water   The thorium filtration curve of Fig 1 shows a maximum at 4×10^{-5} M $ThCl_4$, the concentration at which the membranes are isoelectric   As zeta of reversed sign increases (as indicated by the electroosmosis-concentration curve) the filtration rate falls until in 4×10^{-3} M it is about 22 per cent lower than at isoelectric   The increase in filtration rate as one passes from 4×10^{-3} to 2×10^{-2} M $ThCl_4$ is presumably correlated with a beginning return of zeta toward zero (irregular series) which has been repeatedly demonstrated at high thorium concentrations, we have not carried out electroosmotic observations with 2×10^{-2} M $ThCl_4$ It may be pointed out that the experiments with the stronger thorium solutions are the first to show a decrease in filtration rate below that for water on adding electrolytes, other than those explicable by mechanical plugging   This is as expected, since the mono and bivalent cations employed by other workers could not reverse zeta   The filtration rate is thus an inverse function of zeta, being diminished by an increase of zeta

The question as to which is the more important of the two possible mechanisms, postulated earlier in the paper, by which electrokinetic factors might influence filtration rate is not decisively answered by this work   As regards the electroosmotic back-transport set up by stream potential, it would seem that, even with an increasing zeta as one passes the isoelectric point, the increased conductivity would so diminish stream potentials that this effect would diminish and filtration rate return toward the maximum   If, on the other hand, changes in effective pore diameter due to variations in the rigidity or viscosity of an adsorbed water layer and determined by zeta are the predominant factor, a continued diminution of filtration rate would be expected so

long as zeta is increasing The fact that the latter occurs might be taken as presumptive evidence that the wall layer effect is of more importance than the stream potential-electroosmotic effect However, since the ratio of the conductivity in the membrane with a concentrated solution to that with a dilute solution is probably much less than the ratio of the respective bulk conductivities, one cannot be sure how much the stream potential with the more concentrated solutions is decreased, and in the absence of this knowledge a decision as to the more important of the two factors cannot be made

The experimental findings on filtration rate reported here agree rather well, except at one concentration, with those reported some time ago by Urban and White (1932) The present work was done under better experimental conditions and, where discrepancy exists, the present results are believed more reliable

During the progress of this work Reichardt's paper appeared, in which is developed an equation for calculating the amount of electroosmotic "blocking" due to stream potential The equation is

$$\psi = \frac{2 A^2 d\lambda_0}{B r \lambda},$$

where ψ is the ratio of the volume back-transported electroosmotically to the volume pressed through by hydrostatic pressure, A and B are functions of $\frac{d}{r}$, where d is thickness of double layer and r is pore radius, λ_0 is the von Smoluchowski (electroosmotic) component of surface conductivity per unit cross section of pore, and λ is total specific conductivity in the pore Where ψ is 1, no filtration will occur, where ψ is 0, Poiseuille's law is obeyed We hoped to apply our experimental findings to this equation to see if the observed departures from normal filtration agreed with those predicted by the equation but the evaluation of several of the factors in the equation is so uncertain as to render such an attempt at present premature

In conclusion, some experiments testing a finding of Lepeschkin's may be mentioned Lepeschkin (1933) reported that when filtration rate is plotted against filtration pressure a straight line is obtained which cuts the λ (pressure) axis to the right of the origin This λ intercept, the pressure below which no filtration takes place, was termed

the membrane resistance   We have carried out a number of determinations of filtration rate as a function of pressure   In the earlier experiments results similar to those of Lepeschkin were obtained, shown as the broken lines in Fig 2   It was soon found, however, that this was due to insufficient time for the membrane to come to equilibrium If the rate at a high pressure was determined and the pressure lowered for another determination, several hours were required for the rate to
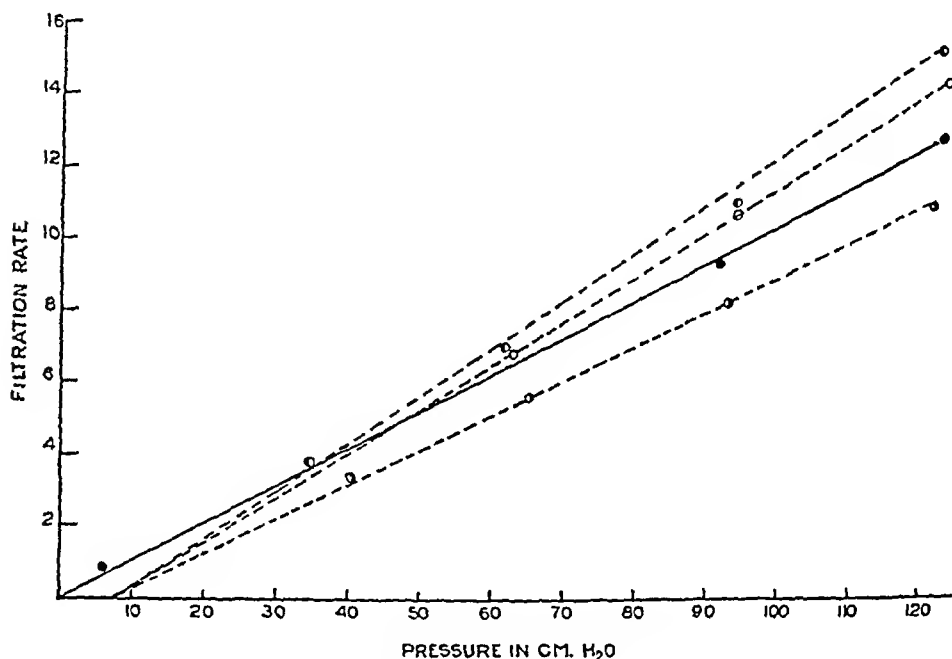
FIG 2   Filtration rate in arbitrary units of $ThCl_4$ solutions through cellophane as a function of pressure   O, water, ◐, 4×10^{-5} M $ThCl_4$ (isoelectric), ◑, 4×10^{-3} M $ThCl_4$, ●, 4×10^{-3} M $ThCl_4$ (pressure equilibrium attained)

become constant   At the lower pressures it was necessary to leave the pressure on overnight to achieve constant conditions   When these precautions are observed the curve is a straight line passing through the origin, a typical result is shown as the full line of Fig 2   We therefore believe that Lepeschkin's membrane resistance is an artifact

SUMMARY

The mechanisms by which electrokinetic factors might influence the filtration rate of aqueous electrolyte solutions through membranes are discussed   The filtration rate of a thorium chloride solution in which

the membrane is isoelectric is compared with those of other solutions The maximum filtration rate is found at the isoelectric concentration, the rate falling as the electrokinetic potential increases

The results demonstrate an inverse relation between the electrokinetic potential and the filtration rate but do not permit the evaluation with any great exactitude of the respective rôles played by the two proposed mechanisms, namely, a stream potential electroosmotic back transport and a variation in effective pore diameter due to an orientation of water dipoles determined by electrical factors

Evidence is presented that Lepeschkin's membrane resistance is an artifact

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A COMPARISON OF THE ELECTROPHORETIC VELOCITIES OF CELLOPHANE AND COLLODION SUSPENSIONS WITH ELECTROOSMOTIC VELOCITIES THROUGH MEMBRANES OF THE SAME MATERIALS

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This paper is a report of the electrophoretic velocities of cellophane and collodion suspensions in ThCl₄ solutions of various concentrations, and of the electroosmotic velocities of the same solutions through cellophane and collodion membranes

EXPERIMENTAL

Cellophane—For the electrophoresis determinations a colloidal suspension of cellophane was prepared by soaking cellophane (previously washed free of glycerin and dried) in a mixture of about equal parts of acetone and ether for several days, putting a few cubic centimeters of this mixture into 100 cc of water and aerating to remove the acetone and ether On two occasions out of several trials satisfactory suspensions were obtained they remained stable for several weeks. We regret that we are unable to specify the factors responsible for the success or failure of this procedure This suspension was examined in a Northrop-Kunitz cell of predetermined cross-section and the current measured It varied from 1×10^{-6} amp in water to 5×10^{-5} in 4×10^{-2} M ThCl₄ The specific resistance of each solution was determined in the usual way and the volts per centimeter across the cell calculated as IR, as suggested by Abramson (1929) Zeta was calculated in millivolts as, zeta $= \dfrac{14 \times \text{micra/sec.}}{\text{volts/cm}}$ The results are shown in Fig 1, where zeta in millivolts is plotted against the negative logarithm of the molar concentration of ThCl₄ The isoelectric point is between 3 and 4×10^{-6} M

The electroosmotic isoelectric point was then determined on intact cellophane membranes A side arm was sealed to a tube of 2 5 cm. bore and 6 cm length and a capillary of 1 mm bore fitted to the side arm A cellophane sheet was tied over one end of the large tube and sealed tight with collodion The upper end of the tube was fitted with a rubber stopper and sealed with beeswax rosin cement. This stopper was perforated by a tightly fitting tube forming an agar KCl bridge

The bridge passed to a large calomel-saturated KCl electrode, its lower end just above the membrane was turned up   The cell was filled with the solution under investigation and dipped into a beaker containing the same solution, the circuit being completed by an agar bridge in the beaker and another calomel electrode
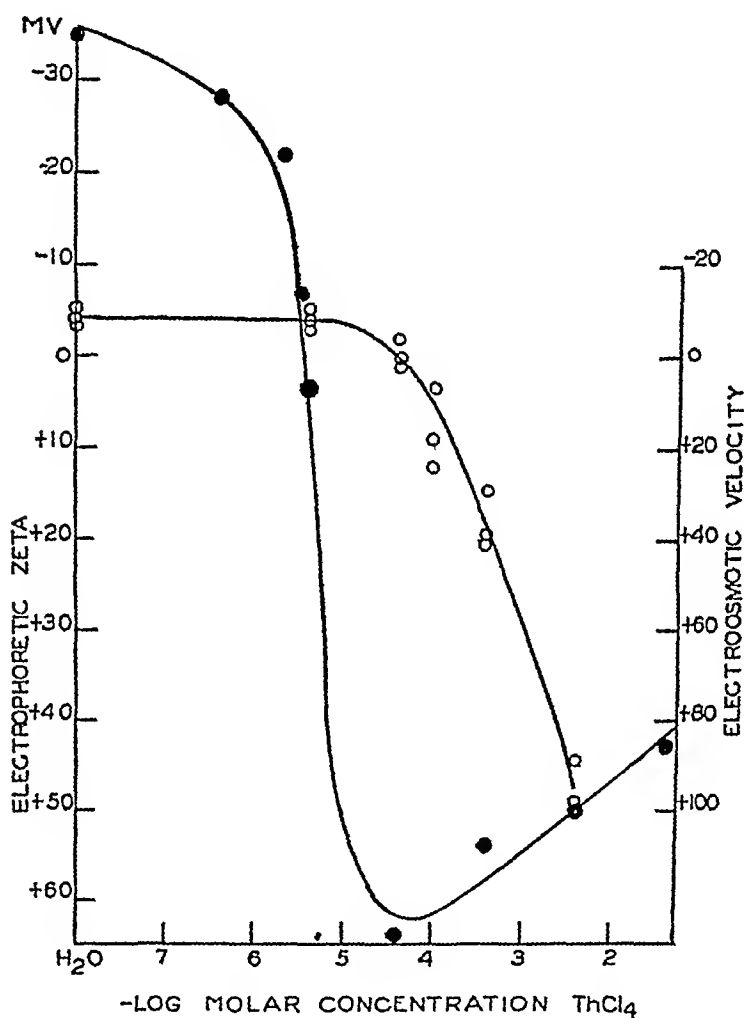
FIG 1 Electrophoretic zeta potentials in millivolts of a cellophane suspension ●, and electroosmotic velocity in arbitrary units of three cellophane membranes O, as a function of the ThCl₄ concentration

The movement of the meniscus in the nearly horizontal capillary side arm was observed with a microscope   100 volts were applied at the electrodes   The current through the cell was always measured   Platinum electrodes were used at first but with these the current was not the same on reversal of polarity, with

calomel electrodes it was unchanged although it rose slowly with time due presumably, to the diffusion of KCl from the agar bridges Higher concentrations than 4×10^{-3} M ThCl$_4$ could not be investigated because of the beating effect of the larger currents The average of several readings with each direction of current was taken The results on three membranes with rate of electroosmotic transport expressed in arbitrary units are given in Fig 1

Absolute values of zeta by the electroosmotic method can be given only if the E.M.F across the membrane is known This must be only a small fraction, in our experiments, of the 100 volts at the electrodes We have attempted to determine this by determining the resistance of cellophane membranes in an apparatus of the type described by Green Weech, and Michaelis (1929) and multiplying by the current We have not yet succeeded, however, in measuring these resistances with consistent enough results to justify a statement as to the E.M.F across the membranes It appears probable that the membrane resistances are so low as not to be measurable with much accuracy It is improbable that the E.M.F across the membrane remains a constant fraction of the applied E.M.F in the various solutions, since the ratio of surface to bulk conductivity must be high and since the diffusion of KCl introduces an inconstant error Therefore, the zeta-concentration curve may not be of the same shape as the electroosmotic transport concentration curve Nevertheless, since the percentile changes undergone by zeta are beyond question much greater than those of E.M.F across the membrane, the transport-concentration curve probably does not greatly differ qualitatively from the zeta-concentration curve In any event, the isoelectric point to electroosmosis is accurately located at 4×10^{-5} M ThCl$_4$ a concentration about 10 times as great as the isoelectric point to electrophoresis The advantage of comparing two processes, as electroosmosis and electrophoresis, by a comparison of their isoelectric points rather than by an evaluation of zeta at values other than zero is that common to all null point methods.

It was thought that this difference in the electrophoretic and electroosmotic isoelectric points on cellophane might be due to inability of the thorium ion to penetrate into the small pores of the cellophane membranes in a reasonable time. That this is not the explanation was shown by the experimental findings that (1) membranes allowed to soak for many days in a concentration of 1×10^{-5} M ThCl$_4$ were still charged as in water (2) actively filtering this solution through a membrane for several hours under pressure did not reverse the sign of charge on the membrane, and (3) even more striking three membranes whose sign of charge had been reversed with a strong thorium solution (1×10^{-3} M) and whose pores must therefore have contained sufficient thorium to bring about reversal very quickly showed a negative zeta potential when placed in 1×10^{-5} M ThCl$_4$

The possibility was then considered that the cellophane suspension used in the electrophoresis study was simply a more soluble fraction of the cellophane membrane, with somewhat different chemical properties from those of the untreated membrane The experiments were therefore repeated on collodion since with this

material suspensions could be obtained which were certainly of the same composition as the membrane
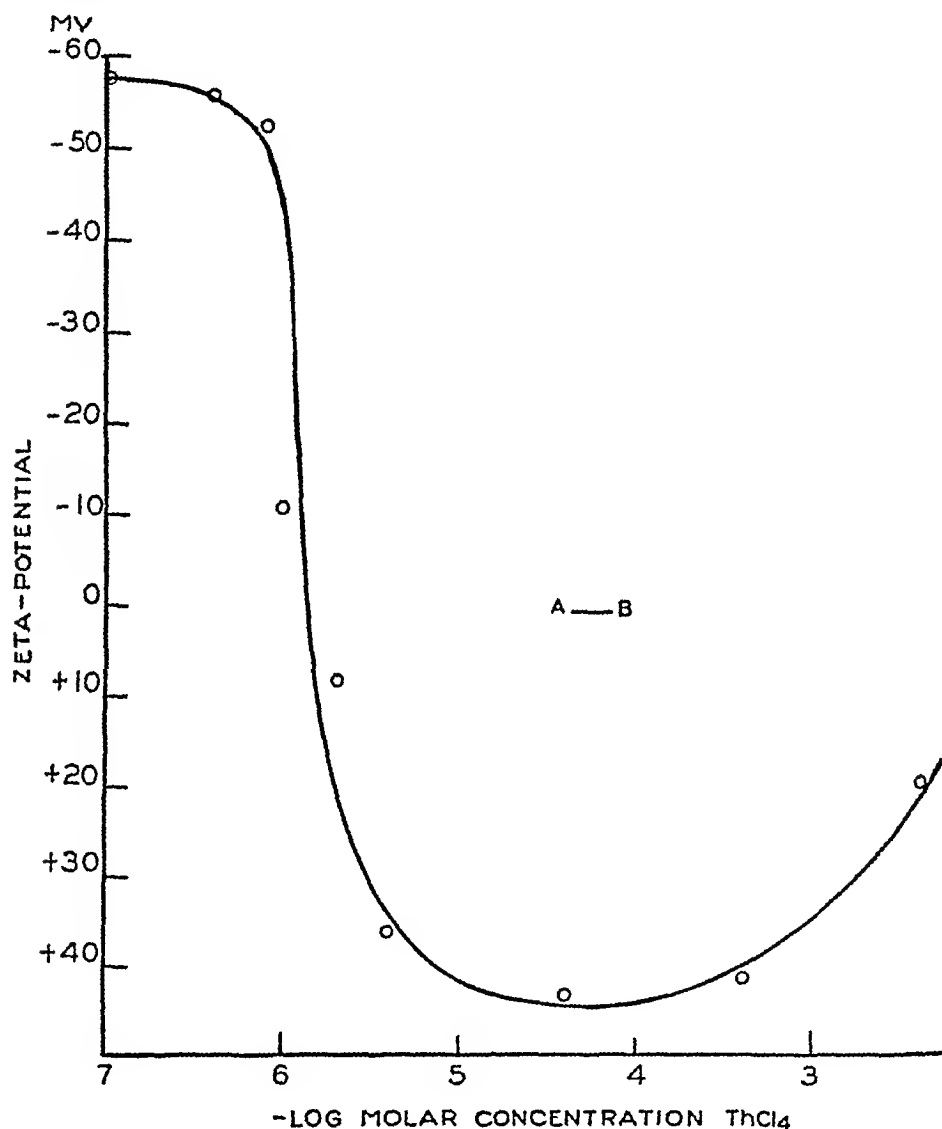
Fig 2 Electrophoretic zeta potentials in millivolts of a collodion suspension as a function of $ThCl_4$ concentration $A-B$ represents the isoelectric zone for electroosmosis in five collodion membranes

Collodion —Collodion membranes of varying pore size were prepared by the method described by Bjerrum and Manegold (1927)   The electroosmotic experiments on the collodion membranes were carried out exactly as with cellophane

Since the membranes differed in permeability (estimated average pore diameter of most permeable membrane between 2 and 3 times that of least permeable) the electroosmotic velocities varied greatly at concentrations other than isoelectric, but the curves for all five membranes crossed the isoelectric point at concentrations between 4 and 6×10^{-5} M $ThCl_4$

Microscopic collodion suspensions for electrophoretic measurements were prepared from the same stock collodion solution used for the preparation of the membranes One part of this solution was diluted with ten parts of the solvent (alcohol and ether) Distilled water was then added slowly, with shaking, until a milky suspension was obtained. The ether and most of the alcohol were then removed by aeration 1 cc of this stock suspension was added to 250 cc of the various thorium solutions under investigation. It should be mentioned that this procedure is not always successful in producing suspensions of the desired particle size (1 to 5μ) often the particles clumped rather rapidly But in about a dozen trials, two suitable suspensions were obtained, these were kept in the ice box and used as a stock suspension for all future electrophoretic determinations

The electrophoretic zeta potential curve and the electroosmotic isoelectric zone are shown in Fig 2 Here again the isoelectric concentration (between 1 and 2×10^{-5} M $ThCl_4$) for the particles is very much less than that found for the membranes (4 to 6×10^{-5} M $ThCl_4$)

DISCUSSION

That the difference in the isoelectric point of cellophane and collodion particles as compared with membranes of the same material is due to the small size of the membrane pores is indicated by the fact that in very large capillaries (300 μ radius) of pyrex glass, or on a flat glass surface, the same concentration of $ThCl_4$ (as well as $AlCl_3$ and $FeCl_3$) which is isoelectric for electroosmosis is also isoelectric for electrophoresis with pyrex particles (Monaghan, White, and Urban (1935))

The behavior of the membranes is probably to be attributed to the influence of the small pores in preventing complete development of the electrical double layers According to McBain and Kistler (1928), the largest pores in cellophane 600 membranes are of the order of magnitude of $2-3 \times 10^{-7}$ cm in radius Let us now consider the probable thickness of the diffuse double layer (for a recent discussion see Müller, 1933) The thickness of the double layer decreases with increasing concentration, the decrease being faster the higher the valency of the ions, according to the expression $\lambda = \dfrac{4\,32 \times 10^{-8}\ cm}{\sqrt{\Sigma z_i^2 \gamma_i}}$ where

λ = double layer thickness, γ_i = concentration in micromols per liter of ions of the 'ith' type, z_i = valence of ions of the 'ith' type This expression holds with a fair degree of accuracy only when $z \zeta \lessgtr 25$ mv
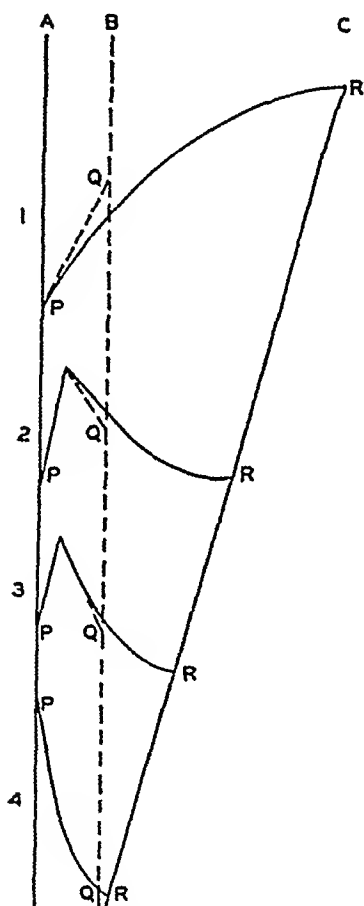
FIG 3 Schematic representation of potential-distance curves in various concentrations of ThCl$_4$ A = solid wall, B = radius of pore in cellophane 600 membranes, C = outer limit of diffuse double layer around a cellophane particle The potential difference PQ = electroosmotic zeta potential of cellophane membranes, PR = electrophoretic zeta potential of cellophane particles Curve 1 represents conditions in water, Curve 2 in 3×10^{-6} M ThCl$_4$, Curve 3 in 4×10^{-6} M ThCl$_4$, Curve 4 in 4×10^{-3} M ThCl$_4$

However, in the absence of data which would permit a more nearly rigorous evaluation of λ we may employ this expression to obtain a rough approximation The calculation yields a double layer thickness

of $5 6 \times 10^{-6}$ cm at the electrophoretic thorium isoelectric concentration (3×10^{-6} M ThCl$_4$) This figure may be several hundred per cent from the true value, nevertheless it seems certain that the double layer thickness at this concentration is much greater than the radius of the membrane pores[1] and consequently that the double layer will be very much compressed in the membrane pores It follows from the condenser equation $\zeta = \dfrac{4 \pi \sigma \lambda}{D}$ that, charge density, σ, remaining the same, ζ is directly proportional to the distance between the plates A schematic representation of the probable course of the potential-distance curves as the thorium concentration is increased is given in Fig 3 (see also Monaghan, White, and Urban (1935)) In water (Curve 1) where the double layer is normally quite diffuse, the zeta potential in the small pored membrane will be greatly reduced from the normal value because of the necessarily compressed state of the diffuse layer In 3×10^{-6} M ThCl$_4$, which is isoelectric for the particles, the membrane (electroosmotic) zeta potential has the same sign as in water (Curve 2) In 4×10^{-5} M ThCl$_4$ (Curve 3) the membrane is isoelectric, while the sign of electrophoretic zeta is reversed In stronger solutions, 4×10^{-3} M ThCl$_4$, where the double layer thickness approaches the pore radius, the potential of the membrane will approach that of the particles, both being of reversed sign (Curve 4)

SUMMARY

It is demonstrated that the isoelectric concentration of ThCl$_4$ is much greater for electroosmosis in small pored membranes (cellophane, collodion) than for electrophoresis of particles of the same material An explanation for the difference is advanced, based on the influence of the small pores in preventing complete development of the electrical double layer

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STUDIES ON BLOOD COAGULATION

I THE RÔLE OF PROTHROMBIN AND OF PLATELETS IN THE FORMATION OF THROMBIN

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When blood is shed, a soluble plasma protein, fibrinogen, is converted into a gel like mass of fibrils in the interstices of which the cellular elements of the blood are retained mechanically to form the blood clot   This may subsequently express serum, the phenomenon of syneresis   Table I represents a condensed and greatly simplified summary of some of the theories which have been suggested concerning the mechanism of this transformation   Without considering them individually or in detail, it is to be noted that according to most of these theories, the conversion of fibrinogen to fibrin is associated with the liberation of an active coagulant, termed thrombin in the absence of any precise information as to its chemical nature   Circulating blood is believed to contain various precursors of thrombin, activators, and inhibiting agents in delicate balance, when blood is shed, this balance is somehow upset, initiating a complex series of consecutive reactions, as the very last step in which thrombin is formed, and this thrombin then reacts with plasma fibrinogen to form fibrin, the basis of the clot

There have been two attempts to turn aside from what we may call these classical theories of coagulation as developed by Schmidt, Hammarsten, Morawitz Bordet Howell (1916–17, 1925) and others with their complex interplay of numerous cellular and plasma constituents, and to substitute instead relatively simple concepts   Thus, Helma has suggested that the transformation of fibrinogen to fibrin is a reversible sol gel transformation, that fibrinogen is the alkaline hydrosol of fibrin, coagulation presumably resulting from an unexplained increase in the

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acidity of shed blood, and that fibrin can be converted back to fibrinogen by dissolving it in alkali   His theory is particularly interesting in view of the fact that Anson and Mirsky[1] have recently succeeded in converting heat-denatured serum albumin back to the native protein by simple solution in acid and careful neutralization   However, Hekma's views have failed of general acceptance   As Barkan and Gaspar showed, an alkaline solution of fibrin is *not* a solution of fibrinogen, for the former cannot be coagulated afresh by the addition of thrombin   Moreover, the coagulation of fibrinogen does not necessarily involve a change in hydrogen ion concentration (Eagle and Baumberger)

Stuber and his coworkers have published an extensive series of papers, in the earliest of which he claimed that thrombin caused coagulation by removing water from fibrinogen, thereby converting it to a dehydrated insoluble form, fibrin   Subsequently, however, he has apparently discarded the classical theories and reactants as artifacts, and has introduced the wholly new idea that coagulation is caused by glycolysis, a concept which has not been verified   As Hartmann and Kuhnau showed, plasma dialyzed free of glucose clots just as readily as normal plasma upon the addition of the necessary electrolytes, and normal plasma may coagulate without any demonstrable glycolysis   Another concomitant of coagulation in the Stuber scheme is an increase in the pH of blood or a fibrinogen solution   Hirsch has reported a *decrease* in the pH of blood during coagulation, while Eagle and Baumberger have been unable to demonstrate any significant change during the coagulation of fibrinogen by thrombin

It would therefore seem that we must revert to the classical theories outlined in Table I as the most promising line of attack on the general problem   If we disregard nosological differences, and seek the basic observations concerning which most of the classical theories are in essential agreement, we find that they all reduce to the following broad formulation   (1) an unidentified substance present in cell-free citrated, oxalated, or untreated plasma (prothrombin), (2) an unidentified substance present primarily in the blood platelets, (3) calcium, and (4) fibrinogen, interact to form an active coagulant, thrombin, and fibrin

Concerning the details of the reaction, however, we find conflicting views in the literature   This and the following paper represent, first, a reinvestigation of some of the controversial aspects of the coagulation phenomenon, and second, further studies of the mechanism of the reaction

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[1] The bibliography follows the second paper of this series, page 553

TABLE I

Some of the Classical Theories As to the Mechanism of Coagulation*

Author	Active participants in the process in addition to fibrinogen and Ca⁺		Inhibitory factors	Supposed sequence of events
	Plasma factor	Platelet factor		
Schmidt (quoted from Morawitz)	Prothrombin	Thromboplastin Zymoplastic substance	Cytoglobin an antithrombin	Prothrombin + zymoplastic substance → thrombin; Thrombin + fibrinoplastic" substance + fibrinogen → fibrin
Wooldridge, and later Nolf (quoted from Morawitz)	(Thrombogen)	Thrombozyme Tissue fibrinogen Thromboplastin		Thrombozyme + plasma factor + Ca + fibrinogen → fibrin + thrombin; fibrinogen → fibrin + thrombin
Morawitz	Thrombogen	Thrombokinase	Antithrombin	Thrombokinase + thrombogen → prothrombin; Prothrombin + Ca → thrombin; Thrombin + fibrinogen → fibrin
Mellanby and later, Pickering (1928)	Prothrombin		An unidentified inhibitory factor stabilizing a prothrombin	Stabilizing factor is somehow destroyed in vitro allowing Ca to activate prothrombin fibrinogen complex and form fibrin
Howell (1916-17 1925) and later Fuchs (1930)	Prothrombin	Thromboplastin Cephalin	Antithrombin Antiprothrombin (heparin) Proantithrombin	Prothrombin antiprothrombin + cephalin → prothrombin + cephalin antiprothrombin; Prothrombin + Ca → thrombin; Thrombin + fibrinogen → fibrin
Bordet	Proserozyme	Cytozyme	A plasma inhibitor affecting serozyme	Proserozyme $\xrightarrow{\text{Ca}}$ serozyme; Contact; Serozyme + Ca → thrombin; Thrombin + fibrinogen → fibrin

* No attempt is made at completeness For general reviews, see Fonio, Howell (1916-17 1925), Morawitz, Pickering (1925 1928), Wohlisch (1929)

† The essential rôle of calcium in coagulation was first recognized by Arthus and Pages

Methods and Materials

1 Fibrinogen —Fibrinogen was prepared from citrated (0 5 per cent) horse, rabbit, dog, or human plasma by four precipitations with 1 2 volumes of saturated NaCl It was sometimes necessary to add a trace of oxalate or citrate to the saturated salt, as otherwise traces of Ca in the salt caused a part of the fibrinogen to coagulate upon redissolving the precipitate A trace of sodium bicarbonate was added to the salt solution to keep the reaction weakly alkaline (pH 7 5–8) After each precipitation, the fibrinogen was collected by filtration (usually overnight in the icebox), redissolved in a minimum volume of 0 85 per cent NaCl, usually 1/2 — 1/4 that of the previous solution, and reprecipitated The final solution failed to clot upon the addition of $CaCl_2$, due to the progressive removal of pro-thrombin and its progressive deterioration in the course of the precipitations. Most of the fibrinogen used in the following experiments was prepared from horse plasma obtained from the Massachusetts State Antitoxin Laboratory, whose courtesy and generosity it is a pleasure to acknowledge

2 Prothrombin and Thrombin —Various methods have been devised for the preparation of these two reagents For the former, Howell (1914) and Cehada precipitated plasma with acetone, dried with ether, and extracted the precipitate with slightly alkalinized water Gratia has inoculated oxalated plasma with staphylococci, the fibrinogen coagulating and leaving the prothrombin in solution In preparing thrombin, both the serum and the clot (Herzfeld and Klinger) have been used In our hands these methods have proved cumbersome or unreliable, and the presence of antithrombin has proved an undesirable complication in using serum as thrombin

Fortunately, a simple method was found[2] which consistently yielded highly active preparations of prothrombin and thrombin within 1 hour after obtaining the plasma Fresh citrated or oxalated plasma, preferably the former, was diluted with 10 to 15 volumes of cold water, and CO_2 gas bubbled through for about 5 minutes A precipitate formed consisting of plasma euglobulin, a portion of the fibrinogen, and a large part of the thrombin precursor, prothrombin (*cf* page 538) The mixture was centrifuged, the supernatant fluid discarded, and the precipitate redissolved in 0 85 per cent salt solution (same volume as the original plasma used) The opalescent solution was brought to approximately pH 7 0, as by the addition of a trace of sodium carbonate, sodium bicarbonate, or very dilute (0 01 N) NaOH [3] By this simple procedure prothrombin was obtained free from serum albumin and free from thrombin-inhibitor (antithrombin) If desired, the fibrinogen was removed by heating carefully at 56°C for 3 to 6 minutes, a procedure which caused

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[2] We have since learned that a similar method was used by Mellanby, who diluted bird plasma with water and acidified with dilute acetic acid, and by Fuchs (1929 *b*)

[3] Phosphate buffer (1/20 volume of M/15 phosphate at pH 8 0) is not as satis-factory, due to the formation of a precipitate upon the addition of calcium salts

no significant destruction of the prothrombin. The coagulated protein was then removed by centrifuging or by filtration through a small coarse filter paper.

To obtain thrombin, one need only add to the prothrombin solution 1/20 its volume of 0 1 M CaCl (1 1 per cent). If the prothrombin had been prepared from untreated plasma, and the fibrinogen had not been removed, the solution clotted in 3 to 15 minutes, whereupon it was broken up thoroughly with a glass rod and filtered through a very small coarse filter paper. The filtrate was a highly active thrombin. If the fibrinogen had been removed by beating the prothrombin solution at 56°C for about 4 minutes, no clot formed and the solution was ready to use as thrombin in 15 minutes after the addition of the calcium salt.

The thrombin solution remained serviceable for 1 to 6 days if kept in the ice box gradually decreasing in activity.

3 Cephalin—Cephalin was obtained from pig brains by drying the tissue with acetone extracting with ether for 3 days evaporating the ether extract to dryness and removing the acetone soluble fraction. The residue was taken up in ether as a 1 or 10 per cent solution, and 0 1 cc. thoroughly shaken in 10 cc. of salt solution to form a 0 01–0 1 per cent suspension. Although the lipoid is referred to as cephalin in the text, it was obviously a crude mixture, containing cephalin lecithin, and probably other lipoids.

4 Platelet Suspension and Platelet Free Plasma—Platelet free horse plasma was prepared by centrifuging citrated plasma for two periods of 1 hour each at 3000 R P M. The clear plasma was then passed through a Berkefeld filter. If an adequate amount of CaCl$_2$ is added to such plasma immediately after filtration, it clots very slowly. After 1 hour however, the plasma becomes completely non coagulable by Ca unless platelets or cephalin are also added.

The sediment of platelets obtained upon centrifuging the citrated plasma was washed twice in a volume of salt solution corresponding to the original plasma volume and resuspended in 1/50 the original volume making a platelet suspension 50 times as concentrated as the original plasma. That such a suspension has not lost its coagulating properties by the manipulation involved in the repeated centrifuging was shown by the fact that the coagulation time of the platelet free plasma was restored to its original value by the addition of 1/50 volume of the concentrated platelet suspension (*cf* page 541).

5 Quantitative Measurement—One of the chief handicaps to the study of coagulation has been the use of qualitative instead of quantitative technique. With this in mind the following quantitative procedure was developed, which, admittedly inexact has nevertheless proved of great value. Suppose it is necessary to compare the thrombin content of a solution before and after a certain physical or chemical manipulation. Keeping all external factors such as temperature volume pH salt concentration etc fixed, and using a constant quantity of all reagents except thrombin one measures the coagulation time in each of a series of tubes containing increasing quantities of thrombin. Given this series as a base line the coagulation time of the unknown solution is then determined under

exactly the same conditions, and the thrombin content obtained at once by interpolation (Table II) The same general method is applicable to every factor entering into coagulation, and is used throughout this and the following paper

The coagulation time is determined by inverting the tube at intervals, gelation sufficient to prevent the contents from pouring being the criterion of coagulation Care must be taken not to shake the contents, for coagulation is thereby accelerated.

Is Thrombin the Cause or Product of Coagulation?

It has been generally believed (Morawitz, Howell (1916, 1925), Bordet (1920 *a,b*)) that coagulation involves two entirely distinct processes the first is the complicated series of reactions culminating

TABLE II

Example of Quantitative Procedure in Estimating the Relative Activity of an Unknown Solution of Thrombin, Compared to a Standard

Original solution of thrombin, *cc* NaCl added to a total of 0 2 cc , 0 8 cc fibrinogen then added	0 2	0 1	0 05	0 025	0 0125	0 0062	0 003
Coagulation time, *min*	1	1½	2½	4	7	12	20
Coagulation time, using the unknown solution of thrombin under exactly the same conditions		3½		10	17		

The thrombin activity of the unknown was therefore about 30 to 35 per cent of that of the original preparation

in the liberation of active thrombin, the second is the interaction of thrombin with fibrinogen to form fibrin However, Wooldridge (quoted from Morawitz), and more recently Nolf, Stuber, and Pickering (1925, 1928) have maintained that thrombin, like fibrin, is a *product* of coagulation rather than its cause, and that the reaction cannot be viewed as two consecutive processes

The prothrombin solution prepared as described on page 534, freed of fibrinogen by heating, made possible a clear-cut test of the two hypotheses If no thrombin forms unless fibrinogen is also added, then the contention of Wooldridge and Nolf gains credence On the other hand, if such a fibrinogen-free solution liberates thrombin upon the addition of calcium, and if the thrombin forms in less than the coagulation time of the original solution, this clearly indicates that thrombin is not a product of coagulation, but its cause As shown

in Protocol 1 the latter is correct Confirming Bordet (1920 a, b) the amount of thrombin formed upon adding $CaCl_2$ to a prothrombin solution is practically the same in the presence or absence of fibrinogen A quantitative study of the velocity of thrombin formation shows that coagulation is coincident with the sudden liberation of large quantities of thrombin, sufficient to cause an almost instantaneous coagulation (< 15 seconds) of the fibrinogen present in the solution

Protocol 1 Showing That the Liberation of Thrombin from Prothrombin is Independent of the Presence of Fibrinogen, and Precedes or Is Coincident with the Actual Coagulation —0 1 cc. of 0 1 M $CaCl_2$ was added to 1 cc. of fibrinogen free horse prothrombin solution (page 534) After 1, 2, 4, 8, 16 and 32 minutes, 0 1 cc. was withdrawn and quickly added to 1 cc of citrated (0 1 per cent) fibrinogen and the coagulation time noted as a quantitative measure of thrombin activity

Time after addition of $CaCl_2$ to prothrombin, min	0	1	2	4	8	16	32	64
Coagulation time of 1 cc fibrinogen plus 0 1 cc of the prothrombin $CaCl_2$ mixture	∞	∞	∞	32	8	3	3	3
Prothrombin converted to thrombin, *per cent*	0	0	0	4	75	100	100	100

The coagulation times were converted to *percentage of thrombin formed* by testing serially decreasing quantities of the final thrombin solution, as follows

Thrombin solution *cc*	0 1	0 05	0 025	0 0125	0 0062
Coagulation time of 1 cc fibrinogen	3	5	8½	14	25

A coagulation time of 5 minutes = 50 per cent thrombin, of 8½ minutes = 25 per cent thrombin, etc

As indicated, between 4 and 8 minutes after adding CaCl to the fibrinogen free prothrombin, there was a sudden liberation of thrombin If Ca salt was added to the same *unheated* prothrombin, containing fibrinogen coagulation occurred in 4½ minutes coincident with the sudden liberation of thrombin and the concentration of thrombin in the fluid expressed from the clot was either the same or less than that formed in the absence of fibrinogen, clear evidence that thrombin is not a by product of coagulation

As was originally believed by Schmidt in 1876 (quoted from Morawitz), and contrary to the contention of Wooldridge (quoted from Morawitz), Nolf, Stuber, and Pickering (1928) the process of coagulation may therefore be separated into at least two distinct reactions

Plasma factor + platelet factor + Ca → thrombin
Thrombin + fibrinogen → fibrin
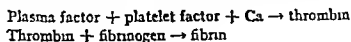
The Rôle of the Plasma Factor, Prothrombin, in the Coagulation Process

The term prothrombin (thrombin precursor) was applied to the plasma factor by Schmidt long before the essential rôle of Ca salts and blood platelets had been discovered   However, the experiments illustrated in Figs 1, 2, and 3 indicate that the term is still applicable, for
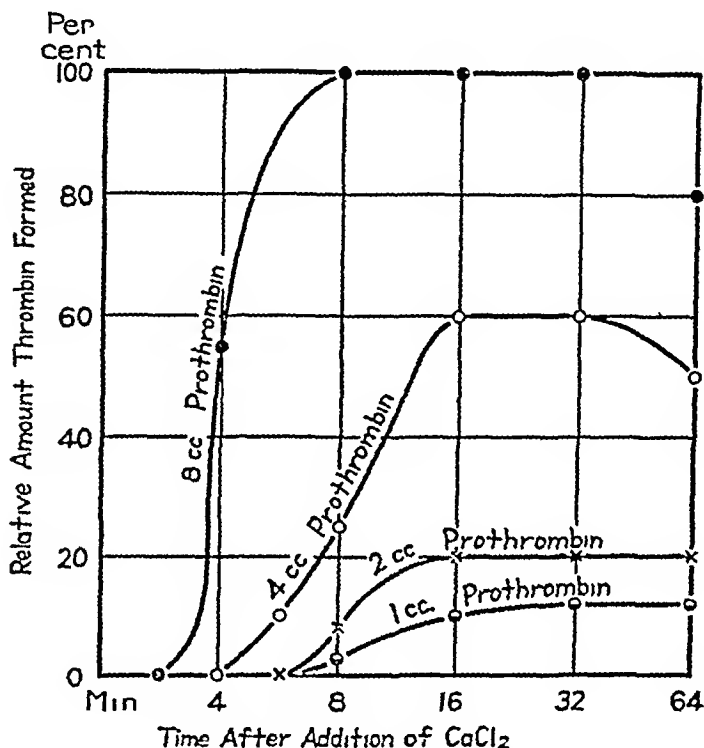
Fig 1 Showing that the amount of thrombin formed depends upon the quantity of prothrombin used   1, 2, 4, and 8 cc of platelet-free prothrombin solution (page 534) + 1 cc of a platelet suspension tenfold concentrated as compared to the original plasma + 1 cc 0 1 M CaCl₂ + 0 85 per cent NaCl to 10 cc   The thrombin formation was followed quantitatively by the method of page 534

the plasma constituent seems to be the true thrombin-precursor, merely activated by Ca and platelets to form thrombin   As is shown in the figures, the amount of thrombin ultimately formed from a mixture of these three reagents is independent of the amount of Ca, platelets, or cephalin used, but varies directly with the amount of the plasma factor   The thrombin was measured by the method described on page 535   Although the figures give the results with horse plasma,

similar results were obtained with dog, rabbit, and human prothrombin, using either a suspension of cephalin or platelets as the activator. The plasma factor has therefore been correctly termed prothrombin, although its chemical nature, like that of thrombin, remains unknown.

We have been unable to confirm its ready adsorption by colloidal Mg(OH) or Ca$_3$(PO$_4$)$_2$ as claimed by Fuchs and Hartmann, in our
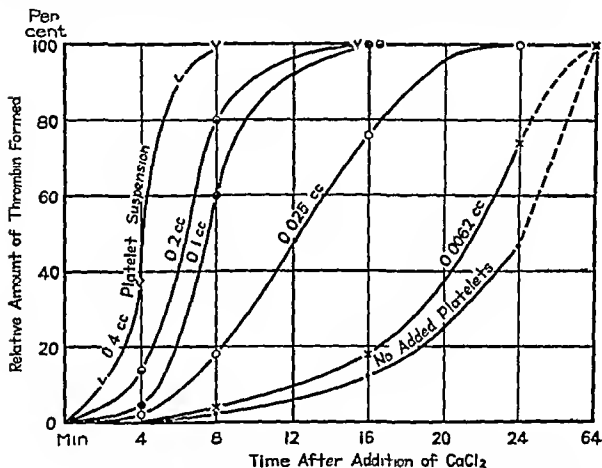
FIG 2 Showing that platelets accelerate the rate of thrombin formation, but do not affect the total quantity formed. 1 cc horse prothrombin solution + varying amounts platelet suspension (20-fold concentrated) + NaCl to 2 cc. + 0 1 M CaCl$_2$ 0 1 cc

hands only a small fraction (< 10 per cent) is so adsorbed, and that is difficult to recover. The identity of prothrombin with complement mid piece as claimed by von Falkenhausen and by Fuchs (1929a,b) is highly questionable. Mid piece, by definition, is that component of the hemolytic factor of fresh serum which is carried down *completely* upon dilution with H$_2$O and slight acidification with e g CO , while, as Bier has shown, prothrombin is only carried down in part by this procedure Indeed, we have found that the supernatant fluid, restored to the proper

pH by the addition of NaOH (approximately 0 1 cc of 1 NaOH per cc serum used), and to proper tonicity by the addition of NaCl (1/19 its volume of 17 per cent solution) clots readily upon the addition of Ca in sufficient quantity to counteract the citrate or oxalate used in preparing the plasma   Such coagulation proves the presence of pro-thrombin, measured quantitatively, about half of the prothrombin
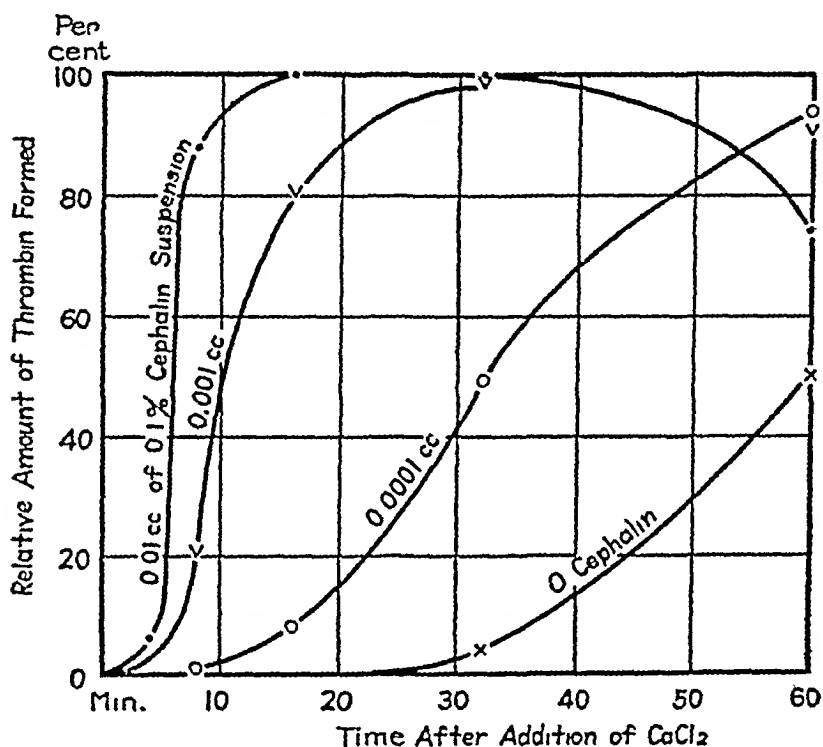
Fig 3 Showing that cephalin accelerates the rate of thrombin formation, but does not affect the total quantity formed   1 cc of horse prothrombin solution (platelet-free) + varying amounts of a cephalin suspension + NaCl to 1 5 cc + 0 5 cc.   x CaCl₂

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The Rôle of the Platelet Factor (Cephalin?) in the Formation of Thrombin

The importance of the blood platelets for coagulation is clearly shown by the fact that even partial removal by centrifuging citrated or oxalated blood at high speed tends to retard coagulation upon subsequent recalcification, and as Cramer and Pringle showed, if the platelets are removed completely, as by Berkefeld filtration, coagulation may be completely prevented. If the latter experiment is repeated quantitatively, one obtains results similar to those summarized in Fig 4. The addition of platelets to Berkefeld filtered plasma causes a progressive and striking increase in the velocity of coagulation upon recalcification, from an initial coagulation time of > 3000 minutes to one of 1–3 minutes. Clearly, the often quoted fact that the blood of patients with purpura hemorrhagica may clot despite platelet counts as low as 20,000 does not disprove their participation in coagulation. In the experiment of Fig 4, the original plasma clotted in 14 minutes upon recalcification, complete removal of the platelets by Berkefeld filtration rendered the plasma non coagulable (*cf* page 535), and as little as 1/20 or even 1/50 of the original platelet concentration sufficed to restore its coagulability. Platelet counts of even 10,000 per c mm represent a quantity adequate to cause a slow but complete transformation of prothrombin to thrombin and of fibrinogen to fibrin.

The use of the terms thrombokinase and thrombozyme for the platelet factor is to be discouraged, inasmuch as these terms imply that it is an enzyme or a coenzyme facilitating the production of thrombin, a hypothesis for which there is some evidence, but which certainly cannot be regarded as proved.

Howell (1912) and Rumpf have shown that a lipoid present in most animal tissue, which both Howell, and Gratia and Levene have identified as cephalin, was an adequate substitute for platelets in the coagulation process. Howell therefore suggested that when platelets disintegrate in shed blood they liberate cephalin, which then participates in coagulation. However, as both Howell and Rumpf have shown, if platelets are extracted with lipoid solvents, only a small fraction (in our hands, less than 10 per cent) of their coagulating activity is recovered in the lipoid solution. Possibly, as Howell has suggested, the active component of platelets is a cephalin protein complex which is more efficient than the cephalin itself.

Numerous workers (Bayne-Jones, Christie, Davies, and Stewart, and Fuchs (1929, 1930) view the platelets not only as a source of a cephalin-like substance, but also as a source of prothrombin This finding, however, we have been unable to confirm In our hands, horse, rabbit, or dog platelets washed free of plasma do not contain significant or even demonstrable quantities of prothrombin, while
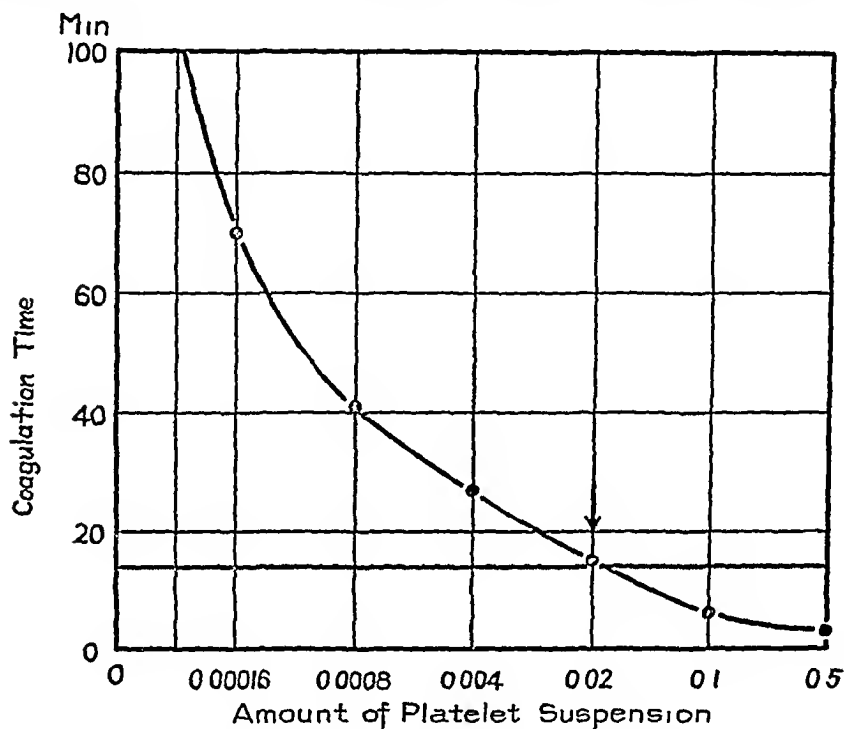
FIG 4 The effect of platelet concentration upon the velocity of coagulation Varying amounts of platelet suspension, 50-fold concentrated as compared to the original plasma, were added to platelet-free plasma prior to the addition of CaCl₂ The original plasma clotted in 14 minutes, as indicated by the arrow in the figure

their coagulating activity as platelets is quantitatively the same as in the original plasma (Protocol 2)

Protocol 2 Showing That Platelets Contain no Demonstrable Quantities of Pro-thrombin —A suspension of washed horse platelets in 0 85 per cent NaCl was pre-pared, 25-fold concentrated with respect to the original plasma (page 535) The coagulation time of a platelet-free horse plasma was restored to normal by the addition of 1/25 volume of this suspension (*cf* Fig 4), indicating that the coagulat-

ing activity of the platelets had not been impaired by the washing To 20 cc of the platelet suspension was added 1 cc of 0 1 M CaCl₂ and the thrombin activity of the whole suspension and of the supernatant fluid tested at intervals (2, 4, 8, 16, 32, and 64 minutes after the addition of the CaCl₂) No demonstrable thrombin appeared while a solution of prothrombin prepared from the same platelet free plasma developed very active thrombin upon the addition of CaCl₂ and a trace of cephalin

Suspending the platelets in H_2O instead of salt solution for 1 hour prior to the addition of CaCl and NaCl in sufficient quantity to restore isotonicity with blood had no effect on the results, and the use of oxalated instead of citrated blood was likewise ineffective Qualitatively the same results were obtained with rabbit and dog platelets In only one instance was prothrombin demonstrated in a horse platelet suspension, after treatment with H_2O and CaCl₂ and in that case the quantity was less than 1/200 that present in the platelet free plasma suggesting indeed that the prothrombin originated from traces of plasma incompletely removed by washing, rather than the platelets themselves Platelets, therefore, have not been demonstrated to contribute significant quantities of prothrombin, their effect in coagulation rests upon their cephalin like activity in accelerating the formation of thrombin

How platelets (or cephalin) accelerate coagulation has hitherto remained an open question The use of a quantitative method discloses the fact that they do not affect the total amount of thrombin produced, but act by accelerating the rate of thrombin production (Protocol 3 and Figs 2 and 3) The more platelets used, the more rapid is the appearance of thrombin and the subsequent coagulation, but the total amount of thrombin formed if the process is allowed to proceed to equilibrium in the absence of fibrinogen depends solely upon the amount of prothrombin used

Protocol 3   Showing That Platelets and Cephalin Accelerate Coagulation by Increasing the Rate of Thrombin Formation, without Affecting the Quantity of Thrombin Produced —Prothrombin was prepared from platelet free horse plasma non-coagulable by Ca (page 534) heated to remove fibrinogen (page 534), and 1 cc was placed in each of a series of tubes Serially increasing quantities of a con centrated platelet suspension (page 535) or of a cephalin suspension were added, the volume adjusted to 2 cc and 0 1 cc of 0 1 M CaCl₂ added to each tube At various intervals 0 1 cc of each solution was withdrawn and quickly added to 1 cc. of horse fibrinogen solution the velocity of coagulation serving as a quantitative measure of thrombin activity (page 535) In plotting the results in Figs 2 and 3, the maximum quantity of thrombin formed has been taken as 100

Similar results were obtained with rabbit and human plasma prothrombin and platelets

amount and rate of thrombin formation in the first reaction are independent of the presence or absence of fibrinogen   After a variable latent period, thrombin suddenly appears in large quantities, coincident with or immediately preceding the deposition of fibrin if fibrinogen is present

3   The amount of thrombin formed in a mixture of prothrombin, Ca and platelets is independent of the platelet or Ca concentration, and depends primarily upon the amount of prothrombin used   The platelets (or cephalin) enormously accelerate the transformation of prothrombin to thrombin, and this acceleration seems to be their physiological rôle in the coagulation process

4   Contrary to previous reports, platelets have not been demonstrated to contain significant quantities of prothrombin

5   The available data do not allow any definite decision as to whether the platelet factor actually combines with prothrombin to form thrombin, or merely catalyzes the transformation   The very slow formation of thrombin in the complete absence of platelets may be due to dissolved traces of platelet material released during the physical manipulation of the plasma (centrifuging, Berkefeld filtration)

6   There was no evidence for a species specific activity of platelets in the transformation of prothrombin to thrombin

(The bibliography for both papers is given at the end of the second paper (page 553))

STUDIES ON BLOOD COAGULATION

II The Formation of Fibrin from Thrombin and Fibrinogen

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The Rôle of Calcium in the Coagulation Process

Two problems with respect to the action of Ca in coagulation are of particular interest Is calcium an intrinsic part of thrombin, and (2) is calcium an intrinsic part of fibrin? Thrombin is not formed from prothrombin unless Ca is present, but as in the case of cephalin, there is some difference of opinion as to whether the calcium actually combines with prothrombin to form thrombin (Pekelharing (quoted from Morawitz), Morawitz, Herzfeld and Klinger) or whether it merely accelerates the transformation According to Loucks and Scott, the addition of citrate or oxalate to thrombin results in a complete loss of activity This, however, we have been unable to verify The calcium in a solution of horse thrombin may be precipitated quantitatively with oxalate without affecting the coagulating activity of the preparation (Protocol 1)

Protocol 1 Showing That the Addition of Oxalate or Citrate to Horse Thrombin Does not Result in Loss of Activity —Thrombin was prepared from horse plasma as described on page 534 to the solution was then added 1/20 volume of 0 5 M sodium oxalate, a fourfold excess as compared with the amount of $CaCl_2$ originally present After 20 minutes, the precipitate was centrifuged at high speed and the supernatant fluid carefully decanted and drained.

Serial quantities of the supernatant fluid were added to 1 cc of citrated solution of horse fibrinogen, and the coagulation times noted A typical experiment follows

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Thrombin, cc , added to 1 cc fibrinogen	0 2	0 1	0 05	0 025	0 0125	0 0062
Coagulation time with untreated thrombin	7	13	23	60		
Coagulation time with oxalated thrombin	7½	15	30	65		

Loss in activity caused by adding oxalate to thrombin solution $= < 10$ per cent

The precipitate was washed once with cold water acidified with CO_2 to minimize re-solution of the Ca-oxalate, redissolved in hot H_2SO_4, and the free oxalic acid titrated in the usual manner with $KMnO_4$ The amount of Ca recovered in the precipitate was, within the limits of experimental error, exactly that originally added to the prothrombin solution

Ca added to 10 cc prothrombin solution 0 5 cc 0 1 m $CaCl_2$ $= 0 00005$ mole
Ca recovered as Ca oxalate   0 000049 mole
                                         0 000053 mole

It should be pointed out that the results of Protocol 1 do not exclude the possibility that thrombin is some sort of a Ca compound, either an ionized compound present in such minute molecular concentration as not to exceed the solubility product of calcium oxalate (e g $<$ 0 00001 m Ca) or, present as a complex salt with a dissociation constant even less than that of Ca-oxalate or Ca-citrate The experiment of Rabinovich, although as yet unconfirmed, is interesting in this connection According to this investigator, electrodialyzed thrombin clots oxalated plasma, but fails to clot electrodialyzed plasma unless Ca salt is added, indicating that the minute quantity of ionic Ca present in the oxalated plasma suffices to activate the formed thrombin, but that Ca is nevertheless essential for the thrombin-fibrinogen reaction In summary, it is an open question whether Ca is an intrinsic part of the thrombin [1]

Much more conclusive data are available with respect to whether calcium is an intrinsic part of fibrin Hammarsten had always maintained that fibrin was not a Ca-protein compound, and cited conclusive analytical data to prove his point Recently however, Mills and Guest have revived the suggestion of Arthus and Pagès that such was the case, that calcium present in thrombin solutions combined stoichiometrically with fibrinogen to form fibrin

[1] Wohlisch and Paschkis seem to have disproved the contention of Vines that the active Ca is part of an organic complex rather than the simple Ca ion

The quantitative relationships here presented seem to confirm the original view of Hammarsten As shown in Protocol 1, it was possible to recover the calcium quantitatively from a thrombin solution without affecting its coagulating activity Even if we allow a 5 per cent error in the determination of calcium, it follows that 10 cc of the oxalated thrombin contained less than 0 1 mg Ca Yet 10 cc of such a thrombin solution clotted 2000 cc of a 0 9 per cent fibrinogen solution to form 16 3 gm of dry fibrin The maximum ratio of Ca to protein in this fibrin was 1 160,000, and unless we assume a molecular weight for fibrinogen of the order of 1,000,000, or unless we make the unlikely assumption that 1 molecule of calcium combines with 50 or 100 molecules of fibrinogen, this excludes stoichiometric combination of calcium with fibrinogen as an essential preliminary to the coagulation of blood If thrombin is a calcium compound, a matter which is still open to question, thrombin and fibrinogen do not react stoichiometrically

The Present Status of the Problem As to the Nature of the Thrombin Fibrinogen Reaction

As in the case of prothrombin, the chemical nature of thrombin is entirely unknown As already indicated, it can be prepared calcium free, within the limits of experimental error, but this fails to exclude the possibility that it is a calcium compound. Whether the platelet factor is an intrinsic part of thrombin, or whether it merely catalyzes its formation, is also an open question (cf page 544 of the preceding article)

The nature of the reaction between thrombin and fibrinogen is unknown Originally the process was believed to be enzymatic, but most of the workers in the field are now inclined to view it as a simple stoichiometric combination between the two reactants We do not believe that the available evidence, summarized in the following paragraphs, justifies any definite conclusion between these two theories, but the weight of evidence does seem to favor the enzymatic theory The pertinent experimental data are as follows

1 A given thrombin preparation may, under favorable conditions, coagulate 1000 times its own dry weight of fibrin For example, 10 cc of a horse thrombin solution (technique of Eagle, page 534) containing 0 21 per cent protein, clotted 3000 cc of a solution of horse fibrinogen

containing 1 52 per cent fibrinogen   The supernatant fluid expressed from the clot contained 0 15 per cent protein   Even if we correct for the possibility that all the thrombin protein is adsorbed in the clot this implies that 21 mg of a thrombin preparation had formed 4100 mg of fibrin   Remembering that these thrombin preparations are very crude, the active constituent probably representing only a minute proportion of the total protein, this implies that thrombin can transform many thousand times its own weight of fibrinogen to fibrin Such a disparity strongly suggests an enzyme reaction

2   It has been claimed as evidence for the stoichiometric combination of thrombin with fibrinogen that there is a linear relationship between the amount of thrombin used and the amount of fibrin formed (Howell (1910), Rettger)   However, as indicated in Protocol 2, and Fig 1, the determination of the quantitative relationships is a very inexact and misleading experiment   With a large excess of thrombin (right side of figure) the fibrinogen is coagulated almost quantitatively, within a few hours, and *the amount of fibrin formed depends solely upon the amount of fibrinogen used*   As less and less thrombin is used, coagulation becomes slower and slower, and equilibrium becomes increasingly difficult to define   If the clot is expressed after 24 hours in the icebox, the supernatant fluid often clots afresh, and fibrin may be formed continuously for as long as 72 hours   By this time more than 75 per cent of the thrombin has deteriorated spontaneously or has been adsorbed by the clot, invalidating the equilibrium results obtained, and with very small amounts of thrombin, coagulation is so slow, and the clots formed so tenuous, that the results are devoid of quantitative significance   In the figure this is indicated by the dashed portion of the curve   The quantitative relationships between thrombin, fibrinogen, and fibrin therefore offer no clue to the nature of the reaction

Protocol 2   The Quantitative Relationships between Thrombin and Fibrinogen — Serially increasing quantities of thrombin were added to each of a series of tubes containing a constant quantity of fibrinogen   Salt solution was added to all the tubes to the same total volume, the tubes were shaken and placed at 2°C for 24 hours   The clots which formed were carefully expressed with a glass rod, and the supernatant fluid replaced at 2°C   The secondary clot was again expressed, and the supernatant fluid replaced at 2°C for a 3rd day   The final supernatant fluids were tested for N content (Nessler reagent, Koch-McMeekin method)   The

difference between the N originally present in the fibrinogen solution and that in the final supernatant fluid gives the amount of fibrin N in the clot The maximum error introduced by disregarding the thrombin N amounts to less than 10 per cent A typical experiment is summarized in Fig 1

3 One of the distinguishing features of enzyme reactions is that the enzyme itself may not be significantly destroyed during the reaction
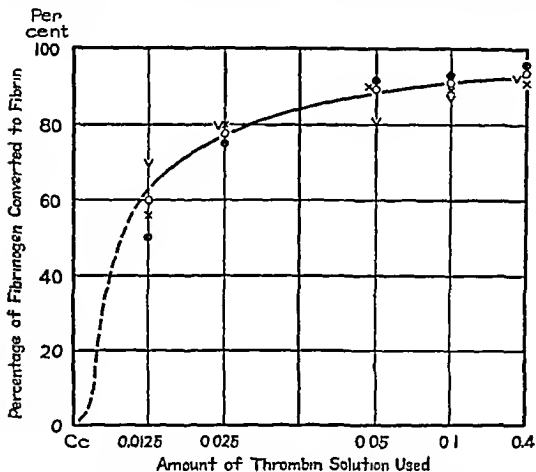
FIG 1 The quantitative relationships between thrombin fibrinogen and fibrin Thrombin solution = 0 099 per cent protein Fibrinogen solution = 1 30 per cent protein Total volume always 9 cc. ● ————— ●, 8 cc fibrinogen solution, O ——————— O, 4 cc , × ——————— ×, 2 cc , v ——————— v, 1 cc

which it accelerates Using a quantitative method of measuring free thrombin (Eagle, page 535), we find that when thrombin and fibrinogen are mixed, there is no decrease in the free thrombin concentration until the very moment of coagulation, when a large proportion suddenly disappears from the fluid, presumably having been taken up into the clot (Fig 2) Klinke observing the process of coagulation nephelo metrically obtained an exactly similar type of curve upon plotting the

degree of turbidity against time, which suggests that the disappearance of thrombin parallels the appearance of visible fibrils   It is to be noted that the amount of thrombin which disappears is many times greater than the minimal quantity necessary to coagulate fibrinogen   The long latent period, the speed with which the disappearance of thrombin proceeds once it begins, and the amounts, involved are difficult to reconcile with either the theory of simple combination between
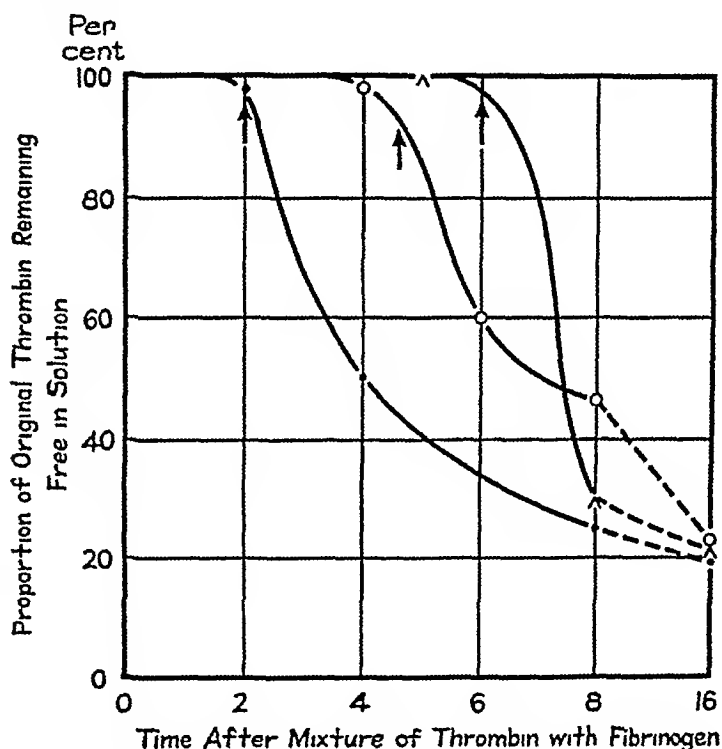
FIG 2 Showing that the disappearance in a thrombin-fibrinogen mixture coincides with the beginning of coagulation   Arrows in the figure indicate beginning gelation

thrombin and fibrinogen or the theory of enzyme action   The thrombin may perhaps be carried down mechanically with the clot

4 Tsunoo has clearly demonstrated that the kinetics of the fibrinogen-thrombin reaction follow no simple equation such as that of bimolecular reaction, and offer no clue to the nature of the reaction As he points out, and as we have been able to confirm, none of the various "formulas" (Fuld) correlating the coagulation time with some

exponential of the thrombin or fibrinogen concentration holds throughout the entire range of concentrations, and none offers any clue to the nature of the reaction

5 Fuld claimed that thrombin and fibrinogen were species specific, that is, that a thrombin derived from a given type of plasma would clot the fibrinogen derived from that same species either exclusively or preferentially   In our hands, however, rabbit, human, dog, guinea pig, or duck thrombin will clot any of these fibrinogens or plasmas, and the velocity of this coagulation offered no evidence of species preference

SUMMARY

Although calcium is essential for the formation of thrombin, it can be recovered quantitatively from formed horse thrombin without affecting its coagulating activity   Citrate also has no significant effect   As stated in the text, this does not exclude the possibility that thrombin is actually a calcium compound present in minute concentration, but confirming the results of Hammarsten, it does show that fibrin cannot be a calcium protein compound unless one assumes molecular weights for fibrinogen greater than 1,000,000

Although the available experimental data concerning the properties of thrombin, the kinetics of its reaction with fibrinogen, and the quantitative relationships between the two do not allow a definite decision as to whether thrombin is an enzyme analogous to rennin, or whether it combines with fibrinogen to form an insoluble compound, fibrin, the weight of evidence does favor the enzyme theory   A given quantity of thrombin can form at least 200 times its weight of fibrin, and in view of the crudeness of the preparation this ratio is probably many times greater   There is no apparent stoichiometric relationship between thrombin and fibrinogen, and thrombin does not disappear from a mixture of the two until the moment of coagulation, the quantity which then disappears is many times the minimal quantity necessary to form the amount of fibrin produced

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QUANTITATIVE STUDIES ON THE PHOTOLETHAL EFFECTS OF QUARTZ ULTRA-VIOLET RADIATION UPON PARAMECIUM

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Relatively few quantitative studies of the photolethal effects of ultra-violet light upon protozoa have been made

Apparently the earliest attempts were those of Dreyer (1903) who found that cysts of *Amebae* were thirty three times as resistant to the carbon arc light passed through a quartz system as were the active forms He also demonstrated (1904) that there is a considerable range of susceptibility of various infusorians to such radiation, and that slight dosages of radiation might kill the infusorians after a lapse of time

Hertel (1905) using a thermopile, determined the relative intensities of mon ochromatic light of each of the following wave lengths 2100, 2320, 2800, 3340 and 3830 A He showed that at equal intensities, the shorter the wave length the greater is its destructive action upon *Paramecium* and that 3340 and 3830 A had practically no effect. Using two approximation methods for determining the absorption of ultra violet light by protoplasm —the fluorescence of the rabbit cornea and the increase in bactericidal time when the cornea is interposed between a culture of bacteria and the beam of light —Hertel concluded that the shorter the wave length the greater the absorption by the cornea and possibly by all cells and added that for a given amount of energy absorbed at any wave length the destructive action is probably the same.

Sonne (1929) and Weinstein (1930) attempted to determine which of the wave lengths of the quartz mercury arc are most destructive to *Paramecium* Both made comparisons on the basis of the total energy delivered per mm^2 to kill Sonne found 2804 A most effective. Weinstein found the wave lengths 2537, 2654 2804 3020 and 3130 A effective to 96 4 100 87 4 20 0, and 11 0 per cent, taking the most efficient wave-length (2654 A) as 100 per cent his incident energy necessary to kill the *Paramecia* varied from 2 162 ergs/mm at 2654 A to 19 629 ergs/mm^2 at 3130 A.

Recently Swann and del Rosario (1932) found that the wave-lengths 2536 and 2894 A killed *Euglenae* readily while 3132 and 3654 A had practically no effect. On their photomicrographs of *Euglenae* taken through a quartz microscope the

nucleus appeared much darker than the cytoplasm at 2536 and 2894 A but indistinguishable from it at 3132 and 3654 A By reading the photomicrographs with a densitometer, they were able to obtain the total extinction coefficients at the given wave-lengths

It has been shown that the lethal effect of quartz ultra-violet radiation on protozoa is strongest at the shorter wave-lengths, apparently becoming quite weak at 3130 A However, the data as to the most effective of the lethal wave-lengths are conflicting and no absorption measurements are available

It, therefore, appeared desirable to the authors first, to attempt to determine the wave-length of maximal photolethal efficiency upon a protozoan cultured in some standardized manner and second, to measure the absorption of the lethal wave-lengths by this animal

Materials

Paramecium multimicronucleata, a large and easily cultured form, was chosen as the experimental animal The clone isolated was grown at 26° ± 0 1°C on a single strain of bacteria in 0 1 per cent lettuce infusion, buffered with 0 0075 M KH_2PO_4 titrated to a pH of 7 0 with NaOH Test tubes containing 15 cc portions of the sterile medium were inoculated with *Pseudomonas ovalis*, and, 24 hours later, twenty *Paramecia* of a healthy clone were added Such cultures showed closely corresponding numbers at comparable times The cultures were used on the 3rd day when vigorous development occurred

A water-cooled quartz-mercury arc giving a constant output at high intensity, running on 2 2 amperes and 250 volts, was used as the source of radiation The monochromator consisted of two crystal quartz lenses and a Cornu equilateral prism 5 cm high and 7 2 cm on a side Both the arc and the monochromator have been described elsewhere (Leighton and Forbes, 1929, Leighton and Blacet, 1932) The exposure chamber is illustrated in Fig 1 Light entering slit S from the monochromator is focused to a parallel beam by lens L (5 cm diameter) and is reflected from the right angle prism P (3 × 2 × 4 2 cm) through the reaction cell C containing the organisms to be irradiated T is the thermopile just above the reaction cell When the thermopile is removed, a low power binocular (20×) can be placed over the reaction cell and the *Paramecia* may be observed in the fluorescent light from the right angle prism

Two reaction cells were used No 1, made by grinding a depression 1 mm deep and 8 mm in diameter in a plate of crystal quartz, was used for lethal dosage studies, No 2, constructed by cementing a piece of glass tubing 6 35 mm in diameter to a crystal quartz plate with chicle and grinding the tubing to a length of 1 mm , was used for absorption measurements Both cells were thoroughly cleansed before using

The line thermopile was constructed by one of the authors the line being approximately 1 mm. wide and 24 15 mm long and containing 20 silver to bismuth junctions, with blackened tin foil radiators, in series It was moved in slot *sl* (Fig 1) across the upper surface of the reaction cell, by turning a screw (millimeter thread) extending to the exterior of the exposure chamber A D'Arsonval high sensitivity galvanometer was used in series with the thermopile The thermopile was calibrated against lamps C 80 and C-81 obtained from the Bureau of Stand ards, and the thermopile factor (22 4 ergs/sec. per mm of beam width per cm galvanometric deflection) was then corrected for the reflection of light from the
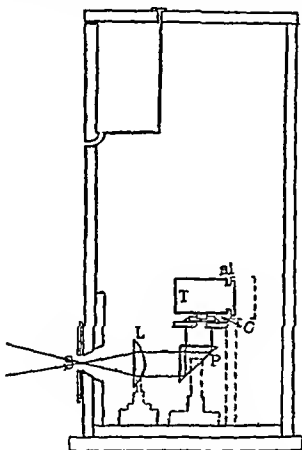
FIG 1 The exposure chamber

quartz window of the thermopile to apply to ultra violet wave lengths (Leighton and Leighton 1932)

Procedure

After the arc had been in operation for half an hour and had attained constancy, an intensity reading was made with the reaction cell No 1 filled with distilled water Similar readings were made at 2 to 3 hour intervals during the day and plotted. The intensity for a given experiment was then read directly from the graph. It was found most convenient to leave the cell uncovered however, a small measured drop of distilled water was added to focus the light upon the thermopile. In the time necessary for intensity readings very little evaporation occurred.

To study the photolethal effects upon *Paramecium*, fifty individuals from a standard culture were transferred into the same reaction cell and culture medium was added until the meniscus was plane (The use of greater numbers of *Paramecia* was found undesirable since it made accurate counts difficult) To prevent evaporation from the surface of the reaction cell, since the exposure lasted from 30 minutes to over an hour, a glass cell was placed over it to form a moist chamber, and the edges of this chamber were periodically coated with water Since only light of wave-lengths shorter than 2000 A produces ozone at atmospheric pressure (Dhar, 1931) it was not thought necessary to seal the *Paramecia* from contact with air

In photolethal studies a problem of great importance is the choosing of an end-point A consideration of the visible effects of light upon *Paramecium* will make clear the most useful criterion of the lethal dose Upon first being irradiated at wave-lengths 2537, 2654, 2804, and 3025 A, the *Paramecia* show slight stimulation, then a gradual decrease in activity accompanied with a shortening and broadening of the individual Next, the contractile vacuoles become huge spheres with the canals enlarged, seemingly jelled, standing out like the rays of a star The cilia of the body then take on an uncoordinated, slow beat and the only movement occurring is a rotation on the long axis due to the beat of the oral cilia The oral cilia may next be inactivated However, vesiculation often occurs before the oral cilia are inactivated and in some cases even while some of the body cilia are still active The vesiculation usually occurs with the formation of a clear vesicle at the posterior end, although at wave-lengths 2537 and 3025 A many vesicles may form When the internal contents of the animal are forcibly ejected into a vesicle, it bursts and the *Paramecium* disintegrates leaving a mass of scattered granules in Brownian movement In the experiments reported here vesiculation was chosen as the criterion of death because it is an end-point easily observed and therefore not subject to so much individual interpretation Furthermore, it is proportional to dosage of irradiation as will be noted from Fig 2

During irradiation, the *Paramecia* were observed periodically (2 minute intervals after the first effects appeared) and the number immobilized or beginning to vesiculate was recorded opposite the time elapsed since the beginning of irradiation

Since during the greater part of the experiment *Paramecia* tend to swim at random on each side of the horizontal midplane of the reaction cell, the intensity of the light at this midplane is a more accurate measure of the average intensity of the light striking the *Paramecia* (The absorption by the twenty five *Paramecia* on the average between front and midplane of the cell can be neglected) Therefore, the
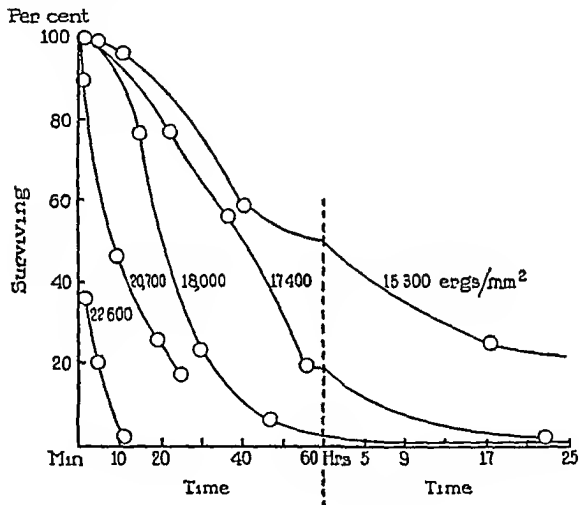
FIG 2 Rate of vesiculation after irradiation (λ 2537 A)

midplane intensity (I_{mp}, the intensity at 0 5 mm depth of culture medium) was calculated by applying Lambert's law

The total energy necessary to produce a given effect consists of two factors—the intensity and the time Theoretically, either one might be varied to produce this effect However, if we keep the intensity constant for different wave lengths, irradiation with light of the less destructive wave lengths will kill only after a longer lapse of time, and, if protoplasmic recovery occurs, the results at different wave-

lengths will not be comparable   Therefore, the time required to produce an effect at different wave-lengths was kept roughly the same, the intensity being varied, the experiments were arranged in such a way as to be completed roughly within 40–60 minutes   To prevent errors which might occur from temperature changes the reaction chamber was maintained at from 25–27°C
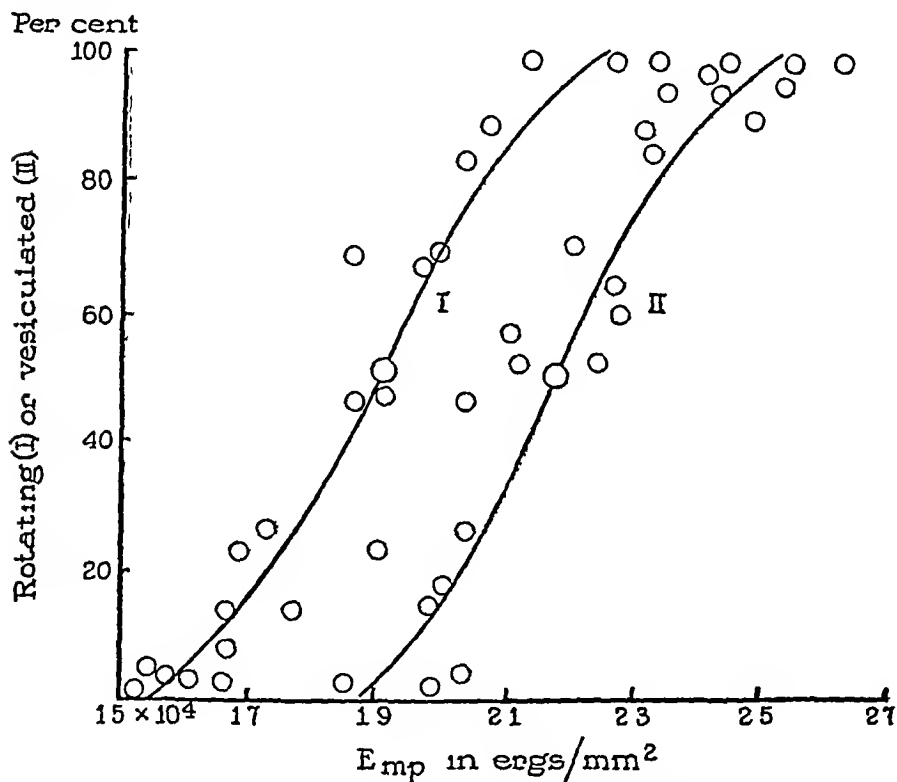
FIG 3   Rotation and vesiculation of *Paramecium* at λ 2537 A

RESULTS

Comparative Photolethal Efficiencies of Different Ultra-Violet Wave-Lengths

Fig 3 gives the numbers of *Paramecia* immobilized and vesiculated for a given amount of irradiation at wave-length 2537 A

Fig 4 shows the relative efficiencies of the wave-lengths 2537, 2654, 2804, and 3025 A in inducing immobilization of body cilia, Fig 5, in inducing vesiculation   In both cases the same sequence is

found 2804, 2654, 2537, and 3025 A   The wave length 3130 A is omitted since preliminary studies showed that it had no destructive effects upon *Paramecia* even after several hours of intense irradiation Both graphs are reproductions of smoothed curves   The standard deviation, σ, of the experimental points from the mean, and the probable error, E_σ, have been calculated and are included on Fig 5   It is
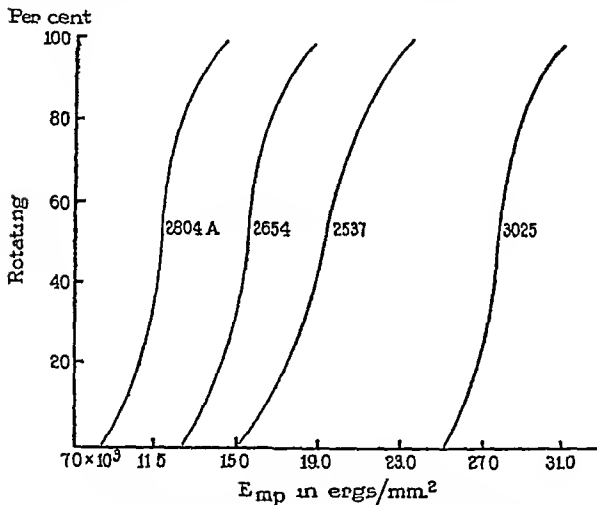
FIG 4   Immobilization of body cilia at λ's 2537, 2654, 2804, and 3025 A

evident that the difference in photolethal efficiencies of the cited wave lengths is many times the largest probable error and several times the deviation from the mean for a given wave-length, therefore the order of effectiveness has statistical significance

Absorption Measurements

While a comparison of the photolethal efficiencies on the basis of the incident energy is interesting, it would be more significant to make the comparison on the basis of the absorbed energy

Although 1 mm of the culture medium absorbs about 20 per cent of the incident radiant energy at the lethal wave-lengths, it is not the agent of the photolethal effect   If the medium is first irradiated with a dosage usually sufficient to kill *Paramecia* and healthy *Paramecia* are then introduced into it, they are in no case injured   In fact, they remain comparable to controls even after 18 hours   Similar results are obtained when healthy *Paramecia* are added to medium in which
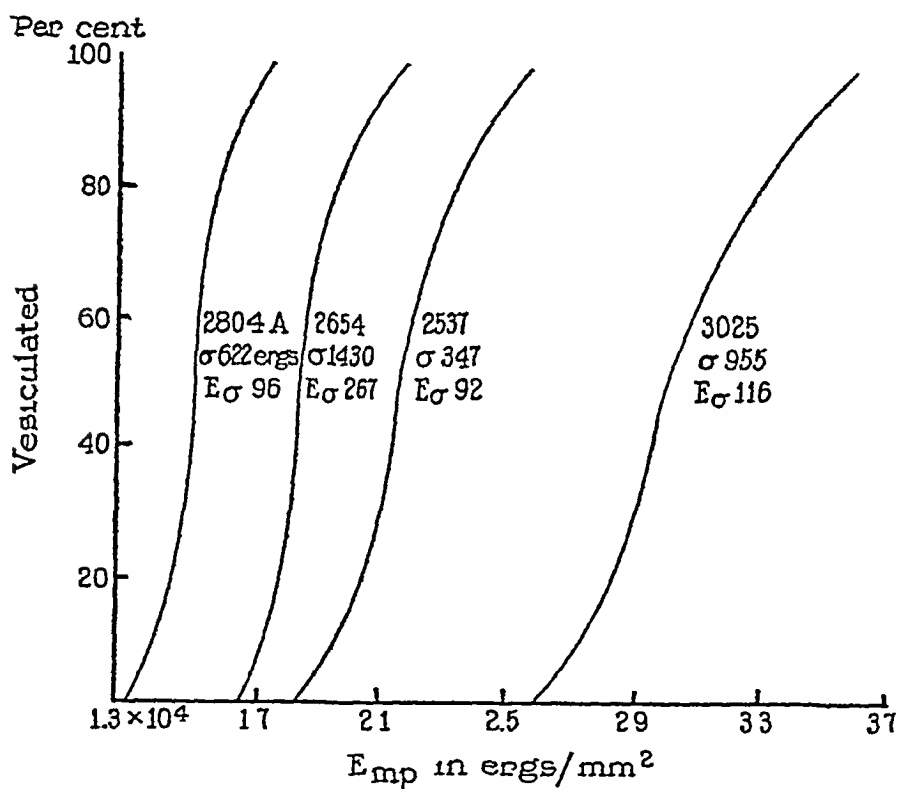
FIG 5   Vesiculation of *Paramecium* at λ's 2537, 2654, 2804, and 3025 A

Paramecia have just been photolyzed   It appears, then, that the photolethal effect is a direct one—due to the absorption of radiant energy by *Paramecium*

The determination of the absorption of ultra-violet light by *Paramecium* offers considerable difficulty since the extinction of ultra-violet light by a suspension of *Paramecia* is due not only to absorption but also to scattering of the light and there is no convenient method

available for measuring this scattering   However, it should be possible
to get an estimate of it by applying a modification of Rayleigh's law
relating scattering and wave length, providing the relative scattering
at several visible wave lengths is known

The scattering in the visible spectrum is readily seen by viewing the *Paramecia*
through a microscope placed at right angles to the beam of light in white light the
Paramecia appear white, in blue light blue, etc. When placed between the visible
light and the eye they appear translucent indicating little, if any, absorption
Therefore the extinction of visible light by *Paramecia* interposed between the
thermopile and the beam of light is due chiefly to scattering

The scattering measurements were sufficiently accurate only when over 1 000
Paramecia were present in the reaction cell This concentration was attained in
the following way A culture was centrifuged for 30 to 60 seconds with a force
equal to about 10 times gravity The supernatant fluid was then drawn off and
balanced salt medium was added after which the *Paramecia* were gently shaken
into suspension After repeating the process the supernatant fluid was withdrawn
and the concentrated suspension of *Paramecia* was transferred to a watch glass
The *Paramecia* were then taken up in a pipette, and, when they had gathered in
dense aggregations at its tip they were pipetted into Cell 2, until a plane meniscus
was obtained A drop of distilled water was then added which served not only
to give the convex meniscus necessary, but also to rouse the *Paramecia* to swim
across the cell thus exposing maximum surface After the measurements had been
taken, the *Paramecia* were removed from the reaction cell, and counted The
total extinction measured divided by the number of *Paramecia* gives the amount
of light extinguished (in visible light) per *Paramecium* The data for illustrative
experiments at 5794 and 4350 A are given in Table I It was necessary to use a
different set of *Paramecia* for each measurement Even with all precautions
taken some *Paramecia* died in every experiment except two As they cytolyzed
they exposed much more surface effective in scattering No correction could be
made for a given number dead as the degree of disruption varied

To calculate the percentage of incident light scattered it is necessary to know
the area occupied by a single *Paramecium* and the number of layers of *Paramecia*
in the reaction cell The effective area of *Paramecium* is approximately the area
of a median sagittal plane This was obtained by diagramming a *Paramecium* to
scale on graph paper and after assigning appropriate units corresponding to the
actual length and width counting the squares in the diagram   In this manner the
area of an average sized *Paramecium* 250 by 65 microns from the 3rd day standard
culture was found to be 0 0106 mm.^{2} As the reaction Cell No 2 had an area of
31 8 mm ^{2}, the *Paramecia* needed to entirely cover its surface with one layer
thickness would be 3,000 As in most of the experiments reported less than half
this number of *Paramecia* were used it can be safely assumed that for most of the
time the *Paramecia* were present as a single layer Only experiments in which

Paramecia remained in constant movement throughout were recorded, when aggregations formed, the data were discarded   The calculated percentages of scattering in the visible light are given in Table I   It will be seen that at 5794 A 18 per cent and at 4350 A 22 per cent of the incident light was scattered

Teorell (1930) showed that for colloidal sols scattering is inversely proportional to the wave-length raised to some power less than 4 (the value assigned by Rayleigh for particles very small in size compared to the wave-length of the light)   It is probable that the scattering by the colloidal particles of the protoplasm of *Paramecium* is similarly related to a lower power of the wave-length   If the ratios of the refractive indices of the protoplasm of *Paramecium* are assumed to be constant at different wave-lengths, it should be possible to obtain an approximate law of scattering by this protoplasm by inserting experimental values for scattering at two visible wave-lengths in the equation

$$\frac{S_1}{S_2} = \frac{\lambda_2^x}{\lambda_1^x},$$

since the various constants would cancel out   Using for S_1 and S_2 the values obtained above for scattering in the yellow and blue light (Table I), one finds $x = 0.7$

Having an approximation of the fraction of the extinction due to scattering, one can obtain the fraction of the extinction due to absorption, by subtracting the scattering from the extinction   The extinction of ultra-violet light was obtained by the method already described for measuring the extinction of visible light   Notwithstanding the considerable error due to cytolysis, the measurements were of the same order of magnitude   Since the scattering corrections are approximate, the data on extinction are sufficiently accurate to show major differences in absorption such as those between the three shorter wave-lengths (2537, 2654, 2804 A) and 3025 A, and between the latter and 3130 A, but not accurate enough to disclose the minor differences between the three shorter wave-lengths   More accurate measurements might be obtained by using the method for determining extinction employed by Swann and del Rosario and correcting for the scattering in the manner herein described   However, a quartz micro-

scope was not available for this purpose   An example of extinction (I_{lp}) at each wave-length and mean values for five such measurements are given in Table I   It will be noted that the order of magnitude of the extinction is similar to that found by Swann and del Rosario for *Euglena*   The absorption (I_{ap}) of light of each wave length was calculated and added to Table I

TABLE I

Absorption by Paramecium

Wave-length	No of Paramecia	I	I_p	I_p	I_p	k_p
$\AA$				per cent	per cent	
5794	2125	58 5	16	16	—	—
			18 ± 0 8	18 ± 0 8	0	—
4350	1250	37 7	24	24	—	—
			22 ± 0 7	22 ± 0 7	0	—
3130	1620	24 8	33	—	—	—
			32 2 ± 1 7	28	4 2	0 02
3025	1750	10 3	44	—	—	—
			42 6 ± 2 2	28	14 6	0 07
2804	2100	4 7	50	—	—	—
			62 0 ± 4 0	30	32 0	0 17
2654	2100	7 7	60	—	—	—
			62 1 ± 6 2	31	31 1	0 16
2537	2160	7 6	60	—	—	—
			65 5 ± 2 0	32	33 5	0 18

The absorption coefficient k_p for a *Paramecium* (a quantity independent of the intensity) may be obtained from Lambert's law

$$\log_{10} I_{lp}/I_p = - k_p \, 1_p$$

if 1_p and the I_{lp}/I_p ratio are known for a single *Paramecium*   Since *Paramecia* were present in a single layer, 1_p is the thickness of a single *Paramecium*, which is fairly constant, varying closely about a mean, for the 3rd day standard culture, and may be ignored in the calcula

tion I_{tp}/I_p can easily be determined as follows   the energy incident upon the *Paramecium*, I_p, could be obtained by multiplying the energy delivered per mm ^{2}, I_o, by the area of a *Paramecium*, 0 0106 mm^2 The intensity of the light transmitted through a *Paramecium*, I_{tp}, is the incident intensity, I_p, minus the absorption, I_{ap}, at the given intensity   The absorption coefficients calculated in this manner for the average intensity and absorption values at each wave-length are given in Table I

The photolethal efficiencies of the lethal wave-lengths can now be compared on the basis of the number of quanta which must be absorbed to produce 50 per cent vesiculation by calculating the energy absorbed per second, multiplying by the time in seconds necessary to

TABLE II

Quantum Efficiency of the Lethal Dose

Wave-length	$A v I_o$	$h v$	I_p ½ ves.	Absorp-tion	I_{ap} ½ ves	Quanta to ½ ves
A	ergs	ergs	ergs	per cent	ergs	
2537	10 3	7 76 × 10⁻¹²	2 31 × 10²	34	7 9 × 10	10 1 × 10¹²
2654	6 2	7 42	1 97	30	5 9	8 0
2804	5 3	7 01	1 63	32	5 2	7 5
3025	12 2	6 55	3 16	15	4 8	7 3

induce 50 per cent vesiculation, and dividing by the quantum for the particular wave-length   The results are summarized in Table II

It will be noticed that the wave-lengths 2654, 2804, and 3025 A are about equally effective, the differences being within the experimental error, while the wave-length 2537 A is less efficient   This lower efficiency of 2537 A may be due to its greater absorption by the surface of the *Paramecium* as suggested by Sonne (1929) or it may be that the difference is only apparent and due merely to the slower onset of the changes leading to vesiculation

DISCUSSION

How does the energy absorbed by the *Paramecium* act in bringing death?   Perhaps the most inclusive theoretical formulation of its possible mode of action is that of Bovie (1918)   The absorption of light by the molecules of protoplasm, he says, results in photo-

chemical changes with the formation of toxic photoproducts Then follow secondary changes which may be due to the reaction between the diffused toxic photoproducts and the materials in various regions of the cell Depending upon the technique employed by the investigators these secondary changes may be detected as changes in permeability, changes in coagulability of the proteins, visible changes, etc Certain parts of protoplasm, because they absorb more radiation (e g parts of the nucleus), or, because their constituents are more susceptible to injury from radiant energy, may be more profoundly affected in the initial reactions than others, and may therefore be the centers of destruction of the cell Sonne (1929), noticing different visible effects of different wave-lengths on *Paramecium*, states that different substances in protoplasm may show preferential absorption and dissimilar reactions at different wave-lengths, e g shorter wave lengths may be so completely absorbed by the surface lipoids that the main effects might be surface phenomena while the longer wave lengths may penetrate the cell and act internally

A number of investigators have attempted to identify the sensitive substances in the cell Harris and Hoyt (1919) thought that since the absorption bands of proteins are due to absorption by tyrosine and phenylalanine, these two amino acids might act as sensitizers, absorbing the radiation and transferring it to other materials in the cell They showed that a solution of tyrosine screened *Paramecia* effectively against lethal ultra violet radiations Burge (1916) attempted to determine whether the enzymes of the cell are the sensitive constituents After irradiating gelatin liquefying bacteria with a dosage sufficient to kill, he ground them up and added the alcoholic extract to gelatin and found that the liquefaction resulting was equivalent to that brought about by living bacteria Gates (1928) suggests that since the absorption by nucleoprotein derivatives at different wavelengths matches the reciprocal of the curves for the relative bactericidal effectiveness of these wave-lengths, the sensitive materials are the nucleoproteins

Blum (1933) has recently suggested that the primary toxic photo product resulting from the absorption of ultra violet radiation may be H_2O_2 He has shown that H_2O_2 is the toxic agent in the photodynamic effect and that thermodynamic considerations support the possibility of formation of H_2O_2 in the ultra violet

To induce vesiculation in *Paramecium* in the experiments here reported, a tremendous number of quanta must be absorbed (Fig 6) In fact, a good proportion of the molecules in a *Paramecium* are probably affected before vesiculation, since some 10^{12} quanta have been absorbed before 50 per cent vesiculation has occurred (Table II) and, on the rough assumption that all the molecules of a *Paramecium* have the average size of an albumin molecule, a *Paramecium* would
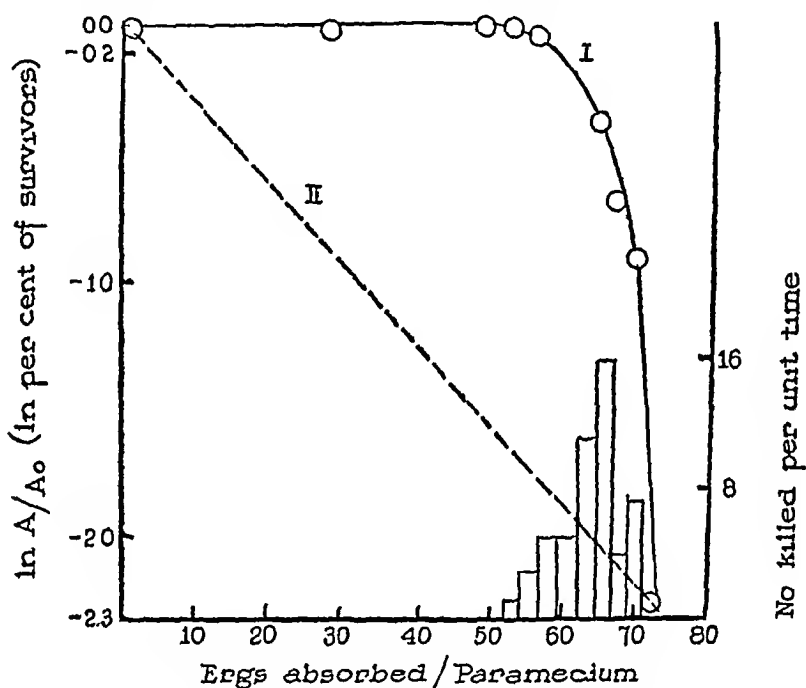
FIG 6 Survival-absorption curve (2654 A)   Curve I, experimental   Curve II, curve expected on the single-hit hypothesis

have some 10^{13} molecules   Of course, certain molecules may absorb many quanta before vesiculation occurs   It is self-evident that the single-hit-to-kill hypothesis advanced for the lethal effects of cathode rays and x-rays upon bacteria (Wyckoff, 1932) is inapplicable for immediate death of *Paramecium,* as a result of irradiation with ultra-violet light

It seems desirable to extend the present work to include a study of the temperature coefficient of photolethal vesiculation, the differential susceptibility of *Paramecia* of different stocks in different states and

under different conditions, the threshold dosage for killing and the sublethal effects of ultra violet light before attempting to add to the analysis of the action of the absorbed light

SUMMARY

Paramecia grown under controlled conditions were irradiated at known intensities of light of wave lengths 2537, 2654, 2804, 3025, and 3130 A   The approximate absorption of the light by the *Paramecia* was found to be greatest and of the same order of magnitude at the three shortest wave lengths, considerably less at 3025, and least at 3130 A

Paramecia did not die when irradiated with high dosages of intense light of wave length 3130 A   At the other wave lengths 50 per cent vesiculation occurred when between 10^{12} and 10^{13} quanta had been absorbed by a *Paramecium*   This would indicate that a very large number of molecules in a *Paramecium* are affected before vesiculation occurs

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THE RELATION BETWEEN CHLOROPHYLL CONTENT AND RATE OF PHOTOSYNTHESIS

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I

INTRODUCTION

This work was undertaken in an attempt to discover whether a quantitative ratio exists between chlorophyll content and the rate of photosynthesis

Plester (1912), working with yellow, light green, and normal varieties of a number of plants, found no direct correlation between the chlorophyll content and the rate of photosynthesis Willstätter and Stoll (1928) working with excised leaves of yellow, etiolated, chlorotic, and normal plants, likewise found no direct relation between the two In neither case were measurements of stomatal apertures made It is therefore possible that in the excised leaves the stomates were not wide open and that the rate of photosynthesis was limited by a deficient supply of carbon dioxide

Likewise, even in chlorotic leaves, the intensity of incident light is greatly diminished by passing through a leaf Consequently all chloroplasts may not be adequately illuminated and light may become a limiting factor In normal or dark green leaves this source of error is even greater because the chloroplasts which are furthest from the surface of incident light may receive very little light This results in a high rate of photosynthesis for the surface chloroplasts and a low rate for the more deeply buried ones The experimentally measured rate of photosynthesis can then be only an average of the two From this it is evident that the work of both Plester (1912) and Willstätter and Stoll (1928) is open to question on the ground of limiting light and possibly of limiting carbon dioxide

Chlorotic leaves are often produced on unhealthy plants having an abnormal metabolism. The photosynthetic rate of chloroplasts in such leaves is probably different than the rate of chloroplasts in healthy leaves Emerson (1929) to avoid as much as possible an abnormal cell metabolism, grew *Chlorella vulgaris* in nutrient solutions lacking iron but containing 1 5 per cent glucose The cells thus produced were chlorotic but by obtaining sugar from the culture medium they grew fairly well. Using graded amounts of iron in a series of cultures he v as able to produce varying amounts of chlorophyll. In any particular series he

found a definite relationship between the chlorophyll content and the rate of photosynthesis   His conclusions are based upon two parallel curves, one containing five points and the other four points   Since so few data were given in his paper and since different series showed such great differences when compared one with another, and since iron may have affected the cell metabolism, this work was undertaken to see if the same relationship existed between chlorophyll content and the rate of photosynthesis when the chlorophyll was varied by other elements such as magnesium and nitrogen   Likewise it was thought desirable to find out if all iron series curves were parallel or whether some were and others were not, and whether there was a definite relationship existing when all curves were plotted on the same sheet

If no other factor is limiting, and if there is some relation between chlorophyll content and rate of photosynthesis, it seems likely that equal amounts of chlorophyll should cause equal rates of photosynthesis

Deficient iron undoubtedly affects the cell metabolism, other than through its effect on chlorophyll   In this work therefore, series were run in which the chlorophyll content was varied by furnishing graded amounts of magnesium and nitrogen as well as iron   In this manner an element not in the chlorophyll molecule, and metallic and non-metallic elements in the chlorophyll molecule were used to control the chlorophyll content   If a direct ratio were found for the iron, magnesium, and nitrogen series, then it would appear as if there were a definite relationship between the chlorophyll content and the rate of photosynthesis

In the correction for respiration, Emerson (1929) states that fluctuations were so slight that a uniform correction of 8 0 mm ^{3} of oxygen per hour for 10 mm ^{3} of cells could be applied   Working at a temperature of 25°C , which was 5° higher than Emerson used, we found that for the same time and volume of cells the respiration varied from 8 0 to 22 0 mm ^{3} of oxygen   The respiration readings of cultures in the same series varied less than those in different series   Emerson (1929) gives a set of respiration readings for a given series which varies from 6 7 to 11 0 mm ^{3} of oxygen   Possibly if he had run respiration determinations on all series, his photosynthetic curves might have been closer together

In this work no correlation between the chlorophyll content and the rate of respiration was found   In some cases the more chlorotic cells had the higher rate and in other cases the greener cells had the higher

rate   Since the rate of respiration varied over such a wide range, it was thought that the most accurate means of correcting for it was to make a separate determination with each culture

The organism used by Emerson (1929) was *Chlorella vulgaris*, while in this work a pure strain of *Chlorella* of undetermined species was used   This culture was obtained through the courtesy of Dr E F Hopkins of the Laboratory of Plant Physiology at Cornell University, and is of the same strain as that used by Hopkins and Wann (1926), and by Hopkins (1930)

<div align="center">II</div>

Method of Varying Chlorophyll Content in Chlorella Cells

A number of elements are cited in the literature as affecting the formation or disappearance of cholrophyll   Various workers have shown that chlorosis may be produced by deficiencies of nitrogen, potassium, phosphorus, calcium, magnesium, sulfur, iron and titanium or by excesses of potassium or chlorine

The stock cultures of *Chlorella* were carried along on dextrose potato-agar slants in test tubes   To inoculate a series a loopful of cells from such a slant was suspended in sterile distilled water and a definite volume of the suspension added to 100 cc of sterile nutrient solution in a 250 cc Erlenmeyer flask with a sterile pipette   A 200 watt Mazda unfrosted bulb was used as the source of light   The culture flasks were arranged in a circle around the bulb at a radius of 21 cm   The bulb was elevated 25 cm above the center of this circle, so that the flasks of culture solutions were about 32 cm from the source of light.   A dull white reflector, 42 cm in diameter was placed above the source of light to diffuse the light more evenly over the cultures   This arrangement gave satisfactory light conditions for the growth of *Chlorella*

Determinations of photosynthesis and chlorophyll content were made after 3 to 6 days of growth, at the end of which time the cells had obtained a good gradation of chlorophyll content   This method produced pure cultures of *Chlorella* which is a necessary prerequisite for a significant determination of photosynthesis   The gradation in chlorophyll was produced by varying the amounts of iron magnesium or nitrogen added to the culture medium

The nutrient solution was that of Emerson (1929) somewhat modified.   It had the following composition

1   *Chlorophyll Gradation Produced by Varying the Iron Concentration*

2 NaC$_6$H$_5$O$_7$ 11 H$_2$O (Baker)	1 00 gm
KNO$_3$ (Baker)	1 26 gm
MgSO$_4$ (Baker)	2 46 gm
KH PO$_4$ (Kahlbaum)	1 22 gm.
Glucose (Kahlbaum)	15 00 gm
Distilled water	1000 00 cc.

This medium contains a slight amount of iron as an impurity but the cultures low in iron gave a sufficiently low chlorophyll content for the purpose of this work Varying amounts of a standard iron solution were added to cultures of the above composition, so as to give iron concentrations varying from zero to two parts per million   The standard iron solution was prepared by dissolving 1 0018 gm of iron wire of 99 84 per cent purity in 10 per cent hydrochloric acid by volume, using heat   Chlorine gas was prepared by adding concentrated hydrochloric acid to crystals of potassium permanganate   This gas was bubbled through the solution in which the iron wire had been dissolved   After the solution had been saturated with chlorine gas, it was evaporated to dryness on a water bath   The residue was taken up in 15 cc of concentrated hydrochloric acid and made up to 1 liter with distilled water   This made a convenient standard solution containing 1 mg of iron per cubic centimeter

2   Chlorophyll Gradation Produced by Varying the Magnesium Concentration

The same nutrient solution was used as for the iron experiments except that sodium sulfate (Baker) was substituted for magnesium sulfate in the ratio of 1 42 gm for 2 46 gm , and that 10 mg of iron per liter were added   This solution contained such a slight amount of magnesium as an impurity, that white cells could be produced in the cultures low in magnesium   A standard solution of magnesium was prepared by dissolving magnesium chloride in distilled water, so that the concentration was 1 mg of magnesium per cc   Varying amounts of this standard solution were added to the cultures so as to give concentrations ranging from 0 02 to 2 0 parts per million

3   Chlorophyll Gradation Produced by Varying the Nitrogen Concentration

The nutrient solution was the same as for the iron experiments, except that potassium chloride (Baker) was substituted for potassium nitrate in the ratio of 0 94 gm for 1 26 gm , and that 10 mg of iron per liter were added   As with the cultures low in magnesium, very low amounts of chlorophyll could be obtained with the cultures low in nitrogen   The standard solution of nitrogen was prepared by dissolving potassium nitrate in distilled water so that the concentration was 10 mg of nitrogen per cc   Varying amounts of this solution were added to the cultures so as to give nitrogen concentrations ranging from 10 to 80 parts per million

III

Determination of the Rate of Photosynthesis

The manometric method used for measuring the rate of photosynthesis and of respiration was developed by Warburg (1919) using the simple or blood-gas manom-

eter of Barcroft and Haldane (1902)   Warburg (1919, 1924, 1931) and Emerson (1929) used this method in their photosynthetic work with *Chlorella*   Descriptions of the method as given by Warburg and Emerson are summarized in the author's thesis (Fleischer, 1933)

In this work the *Chlorella* cells were centrifuged out of the culture medium and suspended in Warburg's carbonate mixture 9 [1]   1 cc. of this suspension containing a known amount of cells, usually from 8 to 15 mm [2], and 6 cc of the carbonate mixture were placed in the vessel, submerged in the thermostat, illuminated, and shaken for an adjustment period of 10 minutes   During this period the stop-cocks must be closed to allow the water vapor in the gas space to come to equilibrium with the carbonate mixture   At the end of the adjustment period the stop-cocks are opened momentarily while the liquid levels in the manometer arms are being leveled and then closed during a run   If the stop-cocks are left open during the adjustment period and closed at the beginning of a run, then the water vapor equilibrium will not have been attained and the vapor pressure of the carbonate mixture will cause an increase in pressure in the manometer, thereby making the rate of photosynthesis appear greater than it really is

During a run the amount of gas evolved for 30 minutes in the light was determined and added to the amount absorbed in the dark during the next 30 minutes, thereby giving the amount of oxygen evolved in photosynthesis

To show that the environmental conditions under which photosynthesis was measured were not toxic to the *Chlorella* cells, determinations were made from several different cultures in which the rate was measured every 15 minutes, for at least 75 minutes

TABLE I

Relation between Time and Amount of Photosynthesis

Time	mm.^{3} of oxygen produced		
	Per 8.0 mm.^{3} of cells	Per 8 0 mm.^{3} of cells	Per 9 2 mm.^{3} of cells
min			
15	21 8	28 0	21 1
15	24 9	31 8	21 3
15	26 6	34 0	22 6
15	25 7	32 8	23 9
15	25 9	33 1	23 5
15	26 1	33 4	
15	25 5	32 6	

[1] Warburg s carbonate mixture Number 9 is composed of 15 cc. of 0 10 mol sodium carbonate plus 85 cc. of 0 10 mol sodium bicarbonate

These data are plotted on the accompanying graph   Since the curves are straight lines they indicate that the rate is constant with time and that no significant toxic effect is evident at the end of 60 minutes

To show that the intensity of light used in photosynthesis was non-limiting, determinations of photosynthesis were made with various dilutions of the same culture   If the weaker dilutions gave a proportionately higher rate of photosynthesis, it would indicate that in the more concentrated suspensions, the cells nearest the light were shading those furthest from the light   But if all of the dilutions gave proportionately the same ratio within the experimental error, then light would not be limiting   The data below are plotted in the accompanying graph and indicate clearly that the intensity of light was great enough to be non-limiting for the process of photosynthesis, since all of the dilutions gave the same proportionate rate of photosynthesis

TABLE II

Effect of Light upon the Rate of Photosynthesis using Various Dilutions of the Same Culture

Time	mm³ oxygen produced per cell volume indicated		
	9 2 mm³	4 6 mm³	3 2 mm³
min			
15	21 1	10 3	4 5
15	21 3	10 9	5 5
15	22 6	10 3	5 5
15	23 9	11 4	5 8
15	23 5	10 5	5 5
mm³ O₂/hr per 9 2 mm³ of cells	89 9	85 4	85 6

The light from a 500 watt Mazda projection bulb, concentrated and made parallel by a plano-convex lens was thrown on the cell suspension through the glass side of the thermostat   The light intensity incident on the Warburg vessels was 75,000 lux as measured by a Weston illumination meter, model 603

The thermostat used was a DeKhotinsky constant temperature bath which maintained the temperature constant to within 0 002°C as checked by a Beckmann thermometer   To correct for changes in atmospheric pressure, a control vessel was filled with 7 cc of carbonate mixture, immersed, and shaken in the water bath, while a photosynthetic run was being made with the other vessels
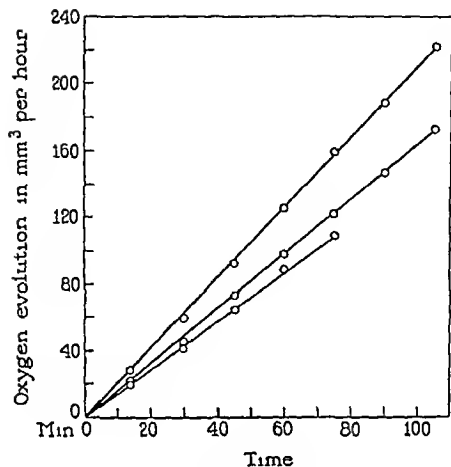
FIG 1 Graph of Table I
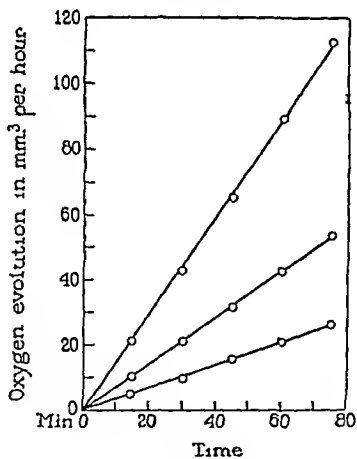
FIG 2 Graph of Table II

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containing *Chlorella* cells   Warburg (1919) used an empty vessel as a control and later (1924) used a vessel containing Ringer's solution   The change in pressure in the control vessel was applied as a correction to the pressure readings of the other vessels to eliminate the effect of changes in temperature or barometric pressure

The volume of gas absorbed in the dark was added to that evolved in the light during an equal period of time, thereby correcting for respiration   The gas volumes were expressed as mm^3 of oxygen per 10 mm^3 of cell volume or per 50 sq cm of cell surface and plotted against their respective chlorophyll content The latter were expressed respectively as gm of chlorophyll per 10 mm^3 of cell volume or per 50 sq cm of cell surface

Cell volume and cell surface were measured as follows   The diameters of 200 cells were measured with an ocular micrometer under an oil immersion lens   The volume and surface of a single average cell were calculated from the diameter The average number of cells per cubic centimeter for five samples of cell suspension was determined with an American standard haemacytometer   The product of the volume of an average cell and the number of cells per cubic centimeter gave the total volume of cells per cubic centimeter of cell suspension   Similarly the total cell surface per cubic centimeter of cell suspension was obtained   The probable error of the method was calculated from the formula

Probable error of a single observation is equal to

$$0\ 6745 \ \sqrt{\frac{V^2 - nM^2}{n - 1}}$$

The probable error as expressed as per cent of the mean was 3 03 per cent

The cell volumes used for determination of photosynthesis were between 8 0 and 15 0 mm^3 of cells as stated above   Since the same type of Warburg glass vessels were used as those employed by both Warburg and Emerson, the cell constants are of the same order of magnitude   In our work the cell constants ranged from 0 49 to 0 60   The manometric readings ranged from 20 to 400 mm showing that enough oxygen was evolved to give a significant reading   Any experimental determination of less than 20 mm was discarded since experimental errors might affect such low readings

IV

Determination of Chlorophyll Content

The various methods of chlorophyll determination as given in the literature are summarized below

Gravimetric —Willstatter and Stoll (1928)

Colorimetric —Maiwald (1923), Schertz (1928), Deuber (1928), Oserkowsky (1932), Guthrie (1928), Sprague and Troxler (1930), Harriman (1930)

Spectrometric —Schertz (1928)

Spectrophotometric —Wurmser and Duclaux (1921), van den Honert (1929), Emerson (1929), Emerson and Arnold (1932)

Spectrographic —Lewkowitsch (1928), Dastur and Buhariwalla (1928), Dastur and Desai (1933)

Spectrocolorimetric —Monteverde and Lubimenko (1913), Hub benet (1925), Zaitseva (1928, 1929), Lubimenko and Hubbenet (1932)

Since this work dealt with chlorotic cells, the amount of chlorophyll to be extracted was too small to be measured gravimetrically Colori metric, spectrophotometric, and spectrocolorimetric readings may be influenced by substances dissolved out of the cells by the extracting solvent Therefore the per cent of error would increase as the chlorophyll content decreased and at very low ranges the determina tion of chlorophyll would be unsatisfactory Likewise since the amounts of chlorophyll to be extracted are so small, it would be ex tremely difficult to separate quantitatively the green pigments from the rest of the plant extract

The spectrographic method was tried using a Bausch and Lomb quartz spectrograph However, the edges of the absorption bands at chlorophyll ranges near the extinction point are not as clear and definite as the results of Dastur and Buhariwalla (1928) would seem to indicate Consequently the position of the curve and its apex point could not be determined sharply

In an attempt to eliminate these sources of error an apparatus was devised whereby the extinction point of an absorption band was determined spectrocolorimetrically A spectroscope was mounted over a single colorimeter tube in which the depth of chlorophyll solution could be varied

Front and side views of the apparatus are shown in Fig 3 *SP* is the spectroscope with *E* the eyepiece and *S* the slit end In all of the work a slit opening of 100μ was used *G* is a Corning filter of heat resisting signal glass No 243 and 3 12 mm thick. This filter absorbs all of the visible spectrum except a band in the red from 6160 Å to 6960 Å The absorption band of chlorophyll used lies in the center of this visible band let through by the glass filter This elimination of the rest of the spectrum allows the observer s eye to concentrate better on the absorption band and to detect with greater ease the presence or absence of the absorption band near the extinction point Since the absorption band is con

trasted with the strips of the spectrum adjacent to the band, the diffusion of light by dissolved or suspended substances does not matter   The absorption band from 6520 Å to 6700 Å used in this work lies in the region of maximum absorption of chlorophyll a and b and outside of the range of absorption of carotin and xanthophyll   Emerson (1929) used this same band in his work

C is the glass colorimeter tube in which the depth of chlorophyll solution may be varied by raising or lowering the plunger P in the tube T O is the three-way stopcock for draining the system at the end of a determination M is a mirror which reflects light from L, a 25 watt internally frosted Mazda bulb, up through the glass bottom of the colorimeter tube

Side view   Front view
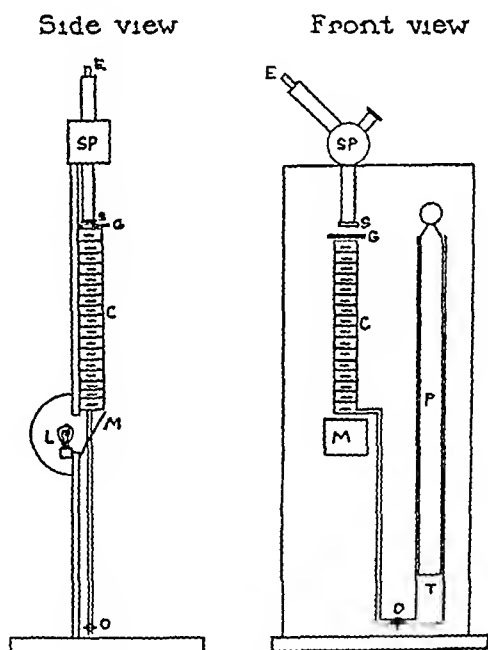
Fig 3 Apparatus for chlorophyll determination

A standard chlorophyll solution was prepared from a sample of pure crystal chlorophyll ($a + b$), obtained through the courtesy of Dr Frank Schertz of the Bureau of Plant Industry, U S Department of Agriculture   This standard solution contained 0 0144 gm of chlorophyll per liter of methyl alcohol   Known dilutions were made, placed in the colorimeter tube, and the depth of solution at which the absorption band just faded out was determined   After several weeks of running such determinations the human eye becomes very sensitive in the detection of this extinction point and fairly good checks could be obtained   In each case at least five depth readings were made and averaged

A set of readings is given below to show the amount of variation The concentrations of chlorophyll are given in percentages of the standard solution and the depths in millimeters at which extinction of the absorption band occurred

TABLE III

Amount of Variation in Determination of Extinction Points

	Standard solution of chlorophyll			
	6 per cent	10 per cent	13 per cent	15 per cent
	mm.	*mm*	*mm*	*mm*
Depths at which extinction oc curred	23 1	15 1	10 6	9 8
	23 8	13 6	11 5	9 4
	23 9	14 6	11 3	9 1
	24 6	13 4	10 6	9 7
	23 1	14 5	10 9	10 0
Average	23 7 mm	14 2 mm	11 0 mm	9 6 mm

For purposes of calculation a column of chlorophyll solution 1 sq cm in cross section was used as a basis To show that the amount of absorption was directly proportional to the concentration of the solution extinction readings were made for a series of dilutions in the same range as that used in the experimental work

TABLE IV

Relation between Absorption of Light and Amount of Chlorophyll

Standard solution	Depth at which extinction occurred	Vol of solution in tube 1 sq cm in area when extinction occurred	Chlorophyll per cc of solution	Chlorophyll in tube 1 sq cm in area
per cent	*mm*	*cc*	*gm*	*gm*
6 0	23 7	2 37	X 0 00000086 = 0 00000205	
10 0	14 2	1 42	X 0 00000144 = 0 00000204	
13 0	11 0	1 10	X 0 00000187 = 0 00000206	
15 0	9 6	0 96	X 0 00000216 = 0 00000207	
Average				0 00000206

Hence when looking through a column of solution 1 sq cm in cross section the absorption band just fades out when 0 00000206 gm of chlorophyll are present in that column of solution regardless of the volume of the solvent To illustrate

Assume that at a depth of 20 mm the absorption band has just faded out

Therefore in a column of that solution, 1 sq cm in area, there would be 2 cc containing 0 00000206 gm of chlorophyll   If there were 50 cc of the original solution, then there would be a total of (0 00000206 × 50)½ = 0 0000515 gm of chlorophyll in that solution   In this manner the chlorophyll content of any solution may be determined

In Table III the variation between any two settings does not exceed 10 per cent However, when the average of five determinations is compared with any other
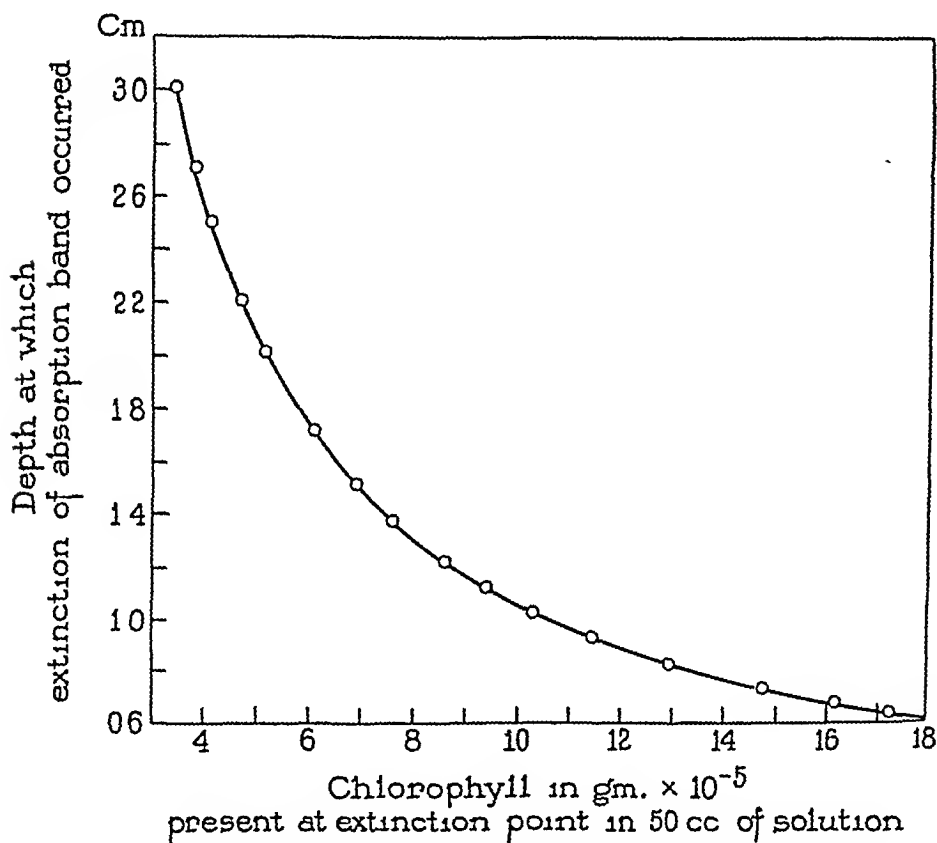
Fig 4   Extinction curve for absorption band between 6520 Å–6700 Å

average as in Table IV, the variation drops to 1 5 per cent   After the preliminary work, an average of ten settings was used so that the variation would be still less This new method of visual determination of the extinction of an absorption band is therefore quite accurate for chlorophyll measurement

To facilitate the computation of the chlorophyll content, the accompanying graph (Fig 4) was constructed   The depth in centimeters at which the extinction of the absorption band occurred is plotted against the grams of chlorophyll present

in 50 cc. of solution at the extinction point   The extinction depth having been determined in the colorimeter, the corresponding amount of chlorophyll may be read off from the graph

Emerson (1929) extracted the pigments from *Chlorella* cells with methyl alcohol but he does not state the technique used   Emerson and Arnold (1932) washed the *Chlorella* cells with distilled water and then poured boiling water over them and let them stand for 2 minutes   The cells were then centrifuged out of the water and extracted with methanol until white   In our work a known amount of cells was boiled in methyl alcohol for 2 minutes, the cells centrifuged to the bottom of the tube, and the clear supernatant chlorophyll solution poured off   The cell residue was suspended in methyl alcohol, again centrifuged, and the superna tant liquid added to the first extract   This solution was made up to a volume of 50 cc with methyl alcohol and the chlorophyll content determined as described above

This method produced pure white cell residues and for completeness and ease of extraction was found to be superior to any of the following

1   Repeated grinding of the cells with a mortar and pestle in 80 per cent or 100 per cent acetone, 95 per cent ethyl alcohol, ethyl ether, or methyl alcohol.

2   Shaking the cells in an International bottle shaking machine for an hour with any of the above solvents

3   Emerson and Arnold's method (1932) as described above

4   Boiling for 5 minutes in any of the above solvents except methyl alcohol

5   A combination of any of the above except boiling in methyl alcohol

To find out whether boiling in methyl alcohol decomposed the chlorophyll, readings were made on three samples of a 15 per cent standard solution of chlorophyll   One sample was left unboiled, the second was boiled for 1 minute, and the third was boiled for 5 minutes   The data below show that boiling in methyl alcohol for as long as 5 minutes caused no significant decomposition

TABLE V

Effect of Boiling in Methyl Alcohol upon the Decomposition of Chlorophyll

Treatment	Depth at which extinction occurred	Vol of solution in tube 1 sq cm. in area when extinction occurred	Chlorophyll per cc of solution	Chlorophyll in tube 1 sq cm in area
	mm	*cc*	*gm*	*gm*
Unboiled	9 6	0 96	$\times$ 0 00000216 = 0 00000207	
Boiled 1 min in methyl alcohol	9 7	0 97	$\times$ 0 00000216 = 0 00000210	
Boiled 5 min in methyl alcohol	9 7	0 97	$\times$ 0 00000216 = 0 00000210	

The differences are within the experimental error of the determination, indicating that boiling in methyl alcohol for 2 minutes, as done in the experimental work, caused no significant decomposition of the chlorophyll

The absorption band used was that within the limits of 6520 Å and 670 Å, when a depth of 43 6 mm of a 13 per cent standard solution was used   This band was then 180 Å wide when light passed through a column of solution 1 sq cm in cross-section and containing 0 00000815 gm of chlorophyll   This solution was considerably stronger than the extinction concentration in order to determine more sharply the edges of the absorption band   At the extinction concentration the band is somewhat narrower and the edges are not so distinct

V

DISCUSSION OF RESULTS

Since all of the series belonging to any one group, for example all of those deficient in iron, were treated identically except for the amount of iron added, it is not necessary to plot each individual series separately   If they are plotted separately, some curves are parallel and others are not, whereas if all are plotted together on the same sheet, a definite linear relationship between chlorophyll content and amount of photosynthesis is evident   This illustrates the danger of using too few data as done by Emerson who has only two curves and nine determinations   In some cases his curves differ by 100 per cent which he does not regard as significant   In our work a total of 104 determinations were made upon which the conclusions are based

A   Experiments with Chlorophyll Gradation Produced by Varied
Iron Concentration

The deficiency of an element may alter the relation between cell volume and chlorophyll content or between cell surface and chlorophyll content and thereby influence the rate of photosynthesis   Therefore the data were calculated so that the results were expressed on a cell volume basis, on a cell surface basis, and on a basis independent of surface or of volume   If the curves are similar, regardless of the manner of expression, then it indicates that the element has not affected the rate of photosynthesis by any effect upon the cell surface or cell volume

It will be apparent that while surface and volume curves might be similar they could not be identical with only a change in scale This latter result is due to variations in cell diameter Chlorella cells which have been grown under identical conditions of light, temperature, and nutrient solution, will exhibit a normal variation in average cell diameter of as much as 5 microns An example will illustrate why these variations prevent surface and volume curves from being identical with only change of scale 10 mm^3 of cells with an average cell diameter of 10 microns have a total cell surface of 60 0 sq cm, whereas the same volume of cells with an average cell diameter of 15 microns have a total cell surface of 39 9 sq cm Although the volumes are equal the surfaces differ considerably Therefore surface and volume curves are not identical with only change in scale, and furthermore, they could not be, unless the average cell diameter were equal

The three different types of graphs, surface, volume, and inde pendent, were made in order to determine whether surface or volume somehow had a specific influence upon the rate of photosynthesis For example if there existed a better proportionality between chlorophyll content and rate of photosynthesis in the surface plotting than in the volume plotting, we might expect that diffusion of oxygen out of or carbon dioxide into the cells was a controlling factor But since all three types of curves were similar it shows that neither surface nor volume play any special rôle in controlling the rate of photosynthesis

The data for the three cases are plotted on the accompanying graphs In each case there is a linear relationship between the rate of photosynthesis and the chlorophyll content indicating that the results are not appreciably influenced by the manner of expression In each case the curve passes through the origin, showing that the rate of photosynthesis does not drop off sharply as the chlorophyll content decreases, but that there is a continuous and steady decrease in both quantities as the origin is approached

In the results of the series given in his paper, Emerson (1929) obtained two curves, parallel but somewhat separated In neither case did his curves approach the origin They also flattened out asymptotically at the higher chlorophyll ranges In contrast to his
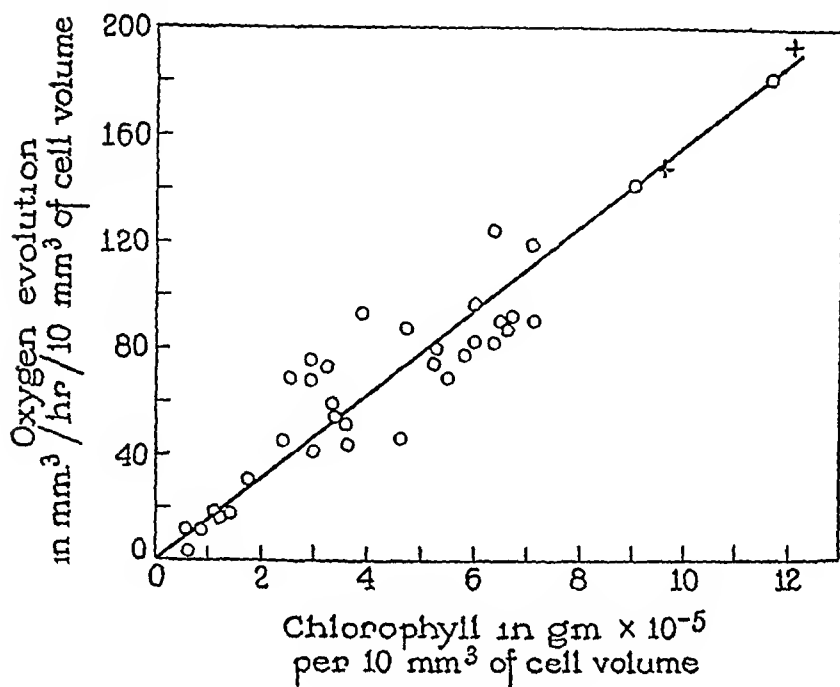
Fig 5   Iron volume plotting
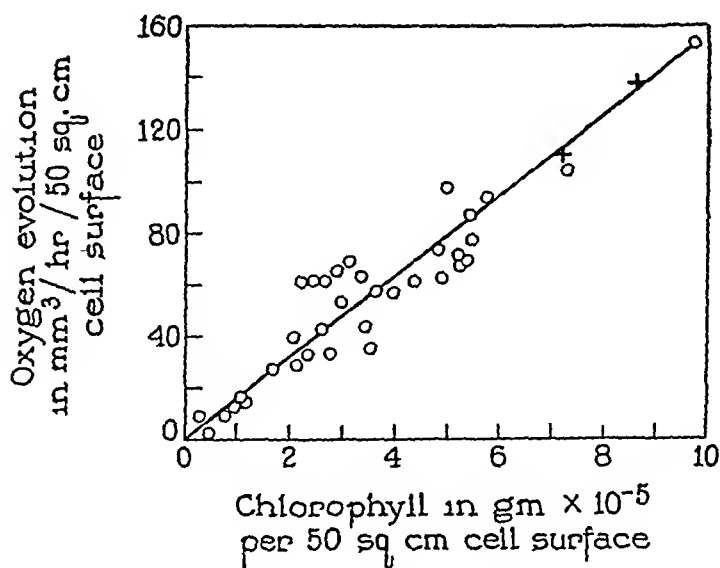
Fig 6   Iron surface plotting

curves, the data here presented form a straight line curve passing through the origin In this work there were three cultures in a series and when curves of different series were compared, some were parallel and others were not But when the results of all of the series were plotted on the same sheet, a linear relationship was evident Possibly if Emerson had run more series he might have found the same situation existing

In order to have a complete range of chlorophyll content, deter
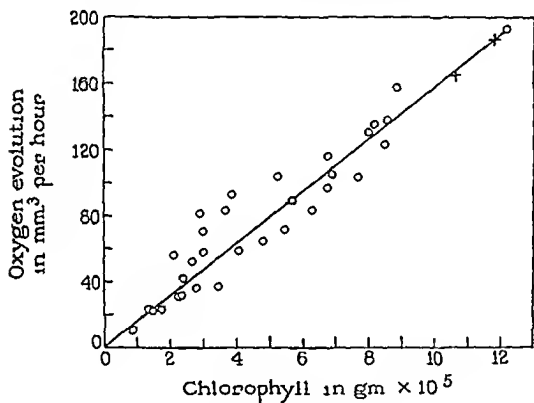
Fig 7 Iron independent plotting

minations were made with cells grown in a full nutrient solution These results are plotted on each of the graphs for iron, nitrogen, and magnesium and the points are indicated by "x" instead of by circles In each of the iron graphs, the full nutrient points lie close to the curve, indicating that even at this high chlorophyll content, chlorophyll is limiting the process of photosynthesis

In none of the results was any correlation found between chlorophyll content and the rate of respiration

The results of the twelve iron series, thirty five cultures in all, given in this paper support Emerson's conclusion that the rate of
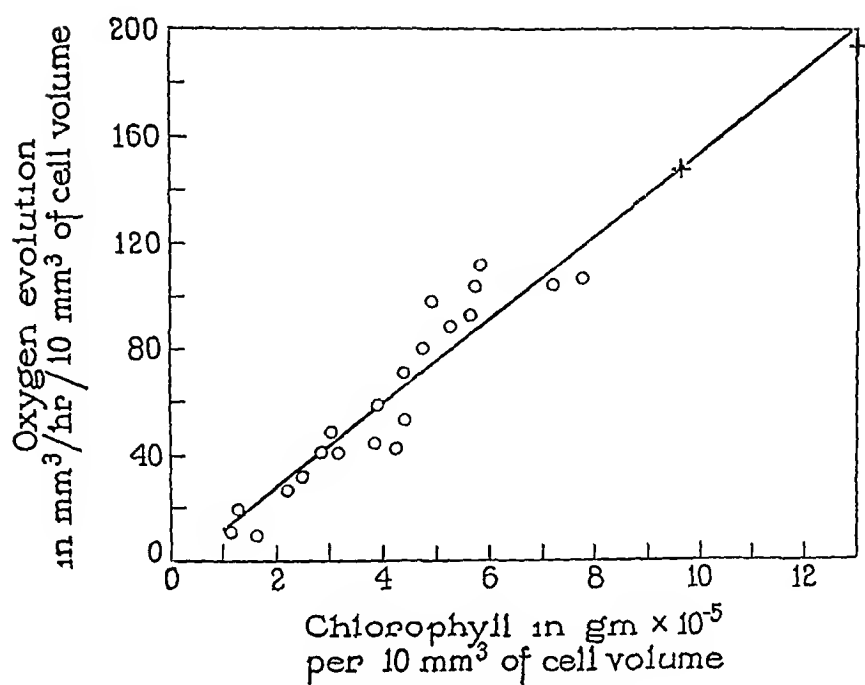
Fig 8 Nitrogen volume plotting
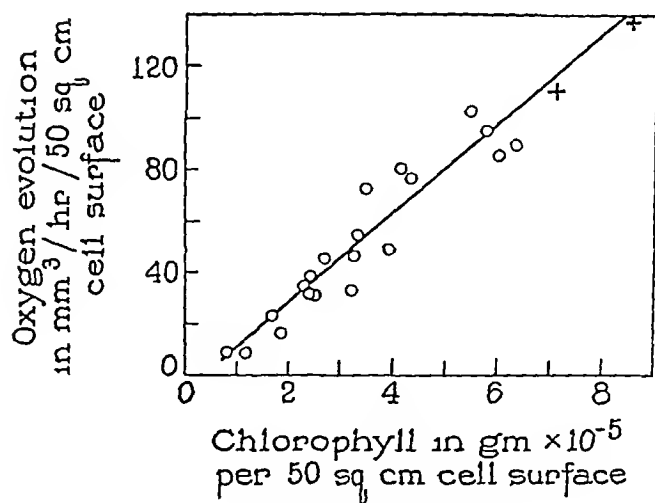
Fig 9 Nitrogen surface plotting
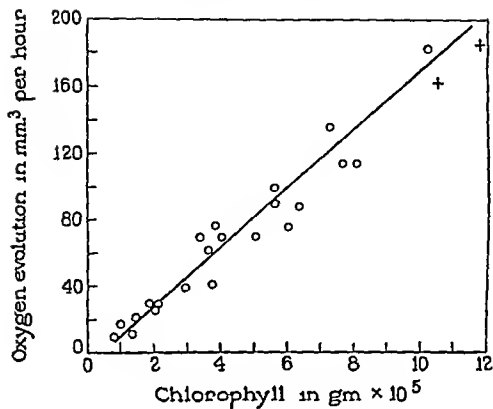
Fig 10 Nitrogen independent plotting
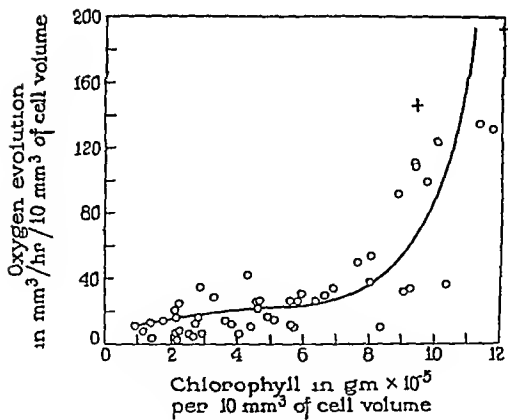
Fig 11 Magnesium volume plotting

photosynthesis is proportional to the chlorophyll content when the latter is varied by varying the iron concentration   However, the
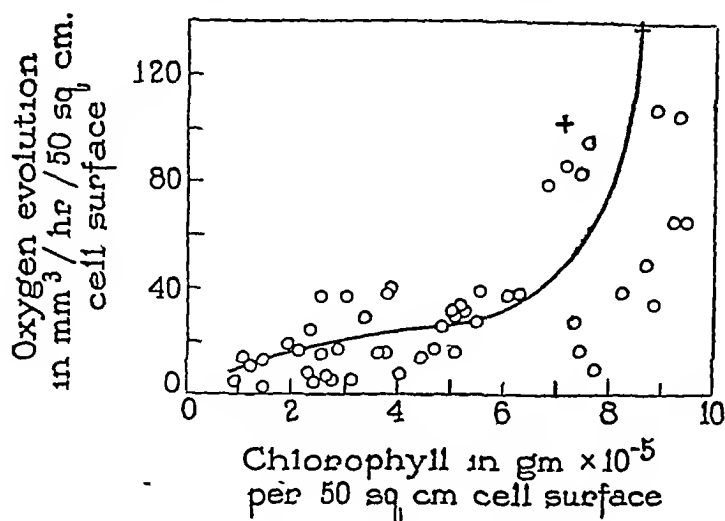
Fig 12   Magnesium surface plotting
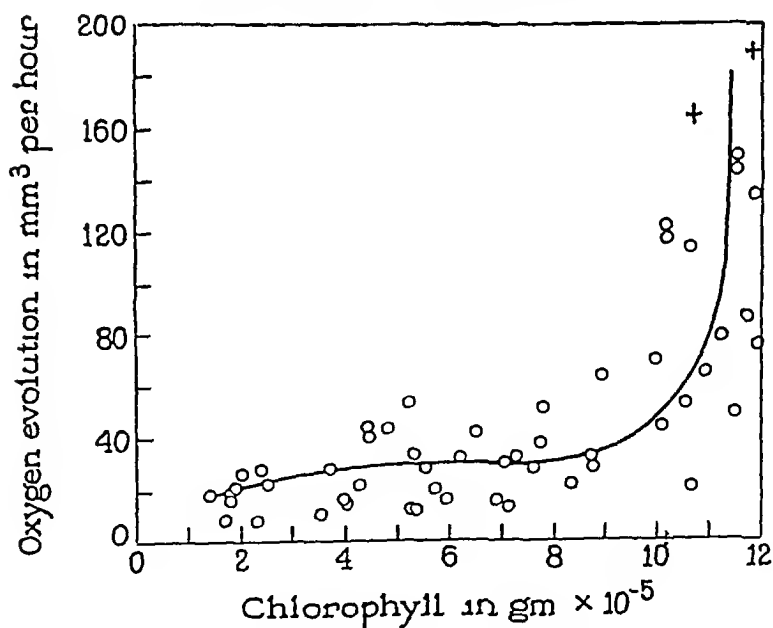
Fig 13   Magnesium independent plotting

curves given here differ from those of Emerson as explained above and appear to substantiate his conclusion better than his own data

do, since his curves do not form a straight line passing through the origin

B Experiments with Chlorophyll Gradation Produced by Varied Nitrogen Concentration

The data for this set of experiments were treated the same as the data for the iron experiments and the results plotted on a surface basis, on a volume basis, and on an independent basis in the accompanying graphs   In each case there is a linear relationship between the rate of photosynthesis and the chlorophyll content   Each curve is a straight line intersecting the abscissa slightly to the right of the origin   Since low nitrogen results in low vegetative growth, this offset may be due to the retardation of photosynthesis by the accumulation of carbohydrates

The full nutrient determination also lies close to these curves, indicating that chlorophyll is still limiting the process of photosynthesis at this high chlorophyll content

The results of the seven nitrogen series, twenty one cultures in all, given in this paper indicate that the rate of photosynthesis is proportional to the chlorophyll content, when the latter is varied by varying the nitrogen concentration

C Experiments with Chlorophyll Content Varied by Varying the Magnesium Concentration

The data for this set of experiments were treated the same as the data for the iron and nitrogen experiments and the results plotted on a volume basis, on a surface basis, and on an independent basis in the accompanying graphs   It was noted that cells, chlorotic through deficient magnesium, were larger than cells which were green and abundantly supplied with magnesium   However, the surface, volume, and independent curves are similar, showing that the greater size of chlorotic cells did not significantly affect the rate of photosynthesis

Although the magnesium curves are similar to each other, they differ from the nitrogen curves and the iron curves   Therefore it appears that the magnesium concentration has an effect upon the rate of photosynthesis separate from its effect through varying chlorophyll content.   The shape of the curves indicates that magnesium becomes

limiting for photosynthesis before it becomes limiting for chlorophyll production   These curves do not approach the origin, which favors the hypothesis that magnesium is doubly involved in the process of photosynthesis

At low concentrations of magnesium the rate of photosynthesis is relatively independent of the chlorophyll content   As the magnesium concentration is increased, the rate of photosynthesis rises rapidly and during the rise is relatively independent of the chlorophyll content Eventually the rate of photosynthesis reaches the value indicated by the full nutrient determinations[2] and at that point the relation between the rate of photosynthesis and the chlorophyll content is comparable to the relation existing in the iron and nitrogen graphs for similar values

The above data indicate therefore, that the presence of magnesium is necessary for the process of photosynthesis in addition to its necessity for chlorophyll formation   Two possible explanations are offered

1 Magnesium is directly concerned chemically or photochemically in the process of photosynthesis

2 Magnesium affects an internal factor other than chlorophyll, this internal factor being concerned in the process of photosynthesis

André (1916) found that the period of greatest photosynthetic activity (April, May, June) was correlated with the greatest amounts of magnesium both organic and inorganic, and also with the greatest organic magnesium/inorganic magnesium ratio   Since his data are given as total amounts rather than as percentages of dry weight, it seems likely that the rapid leaf growth occurring at that time of year would account for the increase in total magnesium   Therefore his data do not prove conclusively that the amount of magnesium present influences the rate of photosynthesis

Since magnesium has a pronounced catalytic effect upon some enzymatic reactions, such as those of phosphotases, it is possible that it may in some similar way catalytically affect the rate of photosynthesis

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[2] Full nutrient determinations are indicated by "x" on the graphs

VI

CONCLUSIONS AND SUMMARY

1 Data are presented which support the conclusion of Emerson (1929) that the rate of photosynthesis is proportional to the chlorophyll content when the latter is varied by varying the iron supply These data give a straight line passing through the origin, which is not true of Emerson's results

2 Similar data are presented which show that a similar relation exists when nitrogen controls the chlorophyll content

3 Evidence is given which indicates that magnesium plays a part in the process of photosynthesis in addition to its effect upon the chlorophyll content

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A PARADOXICAL RELATION BETWEEN ZETA POTENTIAL AND SUSPENSION STABILITY IN S AND R VARIANTS OF INTESTINAL BACTERIA*

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The electrokinetic potential and suspension stability of intestinal bacteria, on the one hand, and biological variation within this group on the other, have both been repeatedly and expertly investigated Nevertheless many phases of the subject remain obscure This obscurity has resulted in part from imperfect understanding of the complexity of the biological material under physical chemical investigation For instance many of the studies antedated the discovery by Arkwright (1920, 1921) that these bacteria occur in two principal variant forms, rough and smooth, whose suspension stabilities differ widely A second major source of confusion is that the characteristic intestinal bacterium has flagella whose physical chemical attributes differ from those of the bacterial body Flagella are abundant on many strains of the typhoid paratyphoid group, are usually sparse on *coli* strains, and are absent from members of the dysentery group The complications introduced by flagella have not even yet found clarification

The present study is concerned with the electrophoretic behavior and suspension stability of four strains, representing the three major groups of intestinal bacteria Each strain has been studied in both its smooth and rough variant form Non flagellate strains were selected as the best way at the moment of avoiding the complications due to flagella

The electrokinetic potential difference (ζ-potential) of the smooth forms has been found to approximate zero over a wide range of electrolyte and hydrogen ion concentrations   This property has not been previously described, so far as we are aware, for other types of suspended particles   Nevertheless these bacteria without measurable ζ-potential form stable suspensions

The rough variants, on the contrary, have electrokinetic potentials which vary with pH and electrolyte concentration in the familiar way Concomitantly these rough forms have values of ζ-potential critical for their suspension stability

Bechhold (1904) observed that typhoid bacilli in an electric field wandered toward the anode   He studied the susceptibility of these bacteria, before and after combination with specific immune serum, to aggregation by electrolytes

Cernovodeanu and Henri (1906) suspended various species of bacteria in distilled water in a moist chamber fitted with two electrodes, the bacteria were observed under the microscope and a weak current was passed through the suspension   Typhoid, colon, and all other bacteria studied, excepting dysentery bacilli, were reported to be electronegative, Flexner dysentery bacilli were thought to be electropositive

Porges and Prantschoff (1906), Buxton and Shaffer (1907), and Buxton and Teague (1907) extended the work of Bechhold

Michaelis (1911) observed that certain bacteria were agglutinable by acid within characteristic ranges of hydrogen ion concentrations   For typhoid bacilli this optimum was approximately $[H^+] = 4 \times 10^{-5}$ Values in agreement with Michaelis were obtained by Beniasch (1911–12), Beintker (1911–13), and von Szent-Gyorgyi (1921)

Beniasch (1911–12) extended the observations of Michaelis to many bacterial species   Strains of *Bact coli commune* and *Bact dysenteriae* were exceptional in that these could not be agglutinated by any concentration of hydrogen ions from neutrality to 10^{-2} Boiled suspensions of the *coli* and dysentery strains were, however, agglutinated at $[H^+] =$ approximately 2×10^{-3}

Arkwright (1914), in a careful study of the acid agglutination of typhoid bacilli, found two optima   The first was at about $[H^+] = 3\ 6 \times 10^{-5}$, loose flocculi were formed, this optimum was abolished by repeated washing of the bacteria, or by heating to 80°C for 30 minutes, or to 100°C for 15 minutes   The second optimum was at $[H^+] = 1\ 1 \times 10^{-3}$ to $2\ 2 \times 10^{-3}$, the clumps were in the form of fine granules, this optimum was not affected by repeated washing or by heating   In the light of later knowledge of the composition of the typhoid bacillus, it seems highly probable that the first optimum was for agglutination of the flagella and the second for the bacterial bodies, although this interpretation seems not to have been subject to experimental test   This would explain the fact, so puzzling to the

early investigators that the bacteria were still negatively charged at the first agglutination optimum

These acid agglutination optima were confirmed by Putter (1921) Putter using a microcataphoresis cell confirmed also the negative charge of typhoid bacilli at the first agglutination optimum

Morishima (1921) produced interesting changes in serum and acid agglutin ability by growing typhoid bacilli continuously in immune serum Though not interpretable at the time these changes seem now to be explainable in terms of ' rough variation and of temporary loss of flagella

Northrop and De Kruif (1921-22) discovered important relations governing the stability of bacterial suspensions Working with the Pfeiffer typhoid strain and a strain of rabbit septicemia bacilli, they measured both the bacterial ζ potential and the cohesive force between bacterial films They found that whenever the ζ potential of the bacteria was less than a certain critical value of about ± 15 mv the bacteria agglutinated provided the cohesive force was not affected If the cohesive force was decreased this critical potential was decreased and if the cohesive force was made very small by high electrolyte concentration no agglutina tion occurred even though the potential was reduced to zero It was further found that all electrolytes tested in concentrations less than 0 01 to 0 1 N affected primarily the potential while in concentrations greater than 0 1 N the effect was principally on the cohesive force

Mudd Nugent and Bullock (1932) formulated suspension stability in terms of probability of collision and of probability of cohesion of collided particles They described strains of intestinal bacteria more hydrophilic than the strains of Northrop and De Kruif such strains although without measurable ζ potential were stable in solutions whose electrolyte concentration was well below that found by Northrop and De Kruif to be necessary to depress the cohesive force Strains of acid fast bacteria were described also which were more hydrophobic than Northrop and De Kruif s strains These bacteria were agglutinated in acid solutions in the presence of electrolyte in concentration higher than 1 0 N

Shibley (1926) described low ζ potentials (3 to 5 mv) for three strains of dysentery bacilli

Brown and Broom (1932) have described typhoid paratyphoid strains virtually without ζ potential Other strains with high negative potential in normal saline have had their ζ potential reduced almost to zero by heating in solutions of salts with monovalent cations no such effect was obtained by heating in the presence of polyvalent cations

Ahuja (1929) failed to detect significant differences between the ζ potentials of rough and smooth typhoid bacilli in distilled water and NaCl solutions

Zylbertal (1931) found an acid agglutination zone of pH = 2 2–3 0 for R strains of *Bact typhosum flexneri shigae coli gallinarum* and *schottmulleri* The S forms of the same series gave no agglutination within the range of pH 2 2 – 5 6

It is evident, then, that many physical-chemical studies have been made to explain the relationship between bacterial suspension stability and ζ-potential   The present study attempts to correlate these suggested relationships with the properties of bacteria as they vary biologically, with the hope of clarifying certain conflicting and confusing findings in the earlier experiments

Materials and Methods

Bacterial Strains Used —A permanently non-flagellate strain of *Bact typhosum*, O 901 (Felix, 1930) long cultivated on artificial media, was used   This strain was smooth in colony form   A rough variant was obtained by Dr C H Hitchcock by growing in broth containing 10 per cent homologous immune rabbit serum, and, alternately, in broth containing the maximum tolerated amounts of lithium chloride   Plates were poured from the broth cultures, and colonies showing change of appearance in the direction of roughness were picked for the next broth culture   This process was continued until the plates showed all rough appearing colonies, which grew in broth as a granular sediment   Serological specificity was incompletely altered as detailed in the paper following (Joffe, 1935)

The strain of *Bact flexneri* used had been isolated 3 years previously from the stool of a dysentery patient   The strain of *Bact coli* was originally of human origin, but had been several years on laboratory media   *Bact pullorum* was a laboratory strain   Rough variants of all these strains were obtained by a procedure like that described for *Bact typhosum* O 901

Bacterial Suspensions —20 to 24 hour broth cultures were killed by adding one drop of formalin to each 10 ml of whole culture and refrigerating for 24 hours, or in other experiments by heating the cultures at 56°C for half an hour   The organisms were centrifuged and washed in 0 85 per cent NaCl twice, and in the solution in which they were to be measured once   After the first washing the organisms were resuspended in the saline, and equal amounts pipetted into individual tubes (100 mm $\times$ 12 mm diameter)   They were then centrifuged, resuspended in test buffer, again centrifuged, resuspended in test buffer, and incubated for several hours at 37°   Final readings were made after an overnight period in the refrigerator   Any sediment was resuspended and electrophoresis measurements were made

Glassware —All glassware was chemically cleaned, thoroughly rinsed in water and distilled water, and dried in air

Buffers —Sörensen's phosphate buffer mixtures were diluted to constant cation concentration   The pK value of a 0 02 M solution was determined (6 91)   Acetate buffer mixtures of constant ionic strength were used   The pK for 0 02 M sodium acetate was determined (4 7)   This is in good agreement with the data of Green (1933)   Clark and Lubs phthalate buffer mixtures were employed in the extreme acid range   All pH values were checked with a quinhydrone electrode, using the value pH = 1 08 for 0 1 N hydrochloric acid

Electrophoresis —The Kunitz (1928) modification of the Northrop-Kunitz microcataphoresis cell was used with a dark field condenser and Bausch and Lomb 8 mm, 0 50 n a, 21× objective At least five readings were taken for each suspension at the two stationary levels *i e*, 0 21 and 0 79 of the inside depth of the cell Radio B batteries were used The applied potential was 125 volts which gave through the cell a potential gradient of 6 5 volts per cm

The cell was kept very clean Before each measurement the cell was rinsed with buffer One drop of bacterial suspension in the same buffer was washed into the cell with 2 mL of buffer Each reading recorded was the total time required for a particle to move a known distance, and, on reversal of the pole-changing switch to return the same distance

The electrophoretic mobility is expressed in micra per second per volt per centimeter drop in potential along the cell If the assumption is made that the dielectric constant and the coefficient of viscosity of the bacteria suspended in the given buffer solution are those of water at 20°C, the electrokinetic potential in millivolts may be calculated from the mobility by multiplication by 12 6 (This assumption as has been previously discussed (Northrop and Cullen, 1921–22, Müller 1933), is not strictly accurate, and becomes less accurate as the amount of salt in solution is changed)

EXPERIMENTAL

In the experiment summarized in Table I, 20 hour broth cultures of the rough and smooth variants of *Bact typhosum*, Strain O 901, were used These were divided into two halves One portion was heated at 56°C for half an hour, the other was not heated Both portions were washed twice in Ringer's solution (pH = 6 8–7 0), once in the buffer measured Electrophoresis data were obtained when the bacteria had been in contact with the buffer for not more than 5 to 10 minutes Immediately after completing the electrophoretic measurements (about 5 more minutes) agar plates were streaked with a loopful of the unheated cultures to determine the viability of the culture

Table I indicates that heated and unheated cultures had the same mobility when subjected to various concentrations of hydrogen ion under the same conditions It also indicates that these strains are easily made non viable in acid buffer solutions

Table II indicates the suspension stability and the electrophoretic mobility for *Bact typhosum* smooth and *Bact typhosum* rough studied by the routine technique The electrophoretic data are plotted in Fig 1

It is evident that this strain in its smooth form had little or no ζ-potential in any buffer measured, whereas in the rough form the

TABLE I

Electrophoretic Mobilities of Heated and Unheated Cultures of Bact typhosum Rough and Smooth in M/50 Buffers of Varying pH

pH of buffer	Bact typhosum S			Bact typhosum R		
	Electrophoretic mobility		Viability of unheated cultures	Electrophoretic mobility		Viability of unheated cultures
	Heated	Unheated		Heated	Unheated	
	μ/sec /volt/cm			μ/sec /volt/cm		
5 3	0 0	0 0	Growth	−2 05	−2 0	Growth
4 7	0 0	0 0	Very slight growth	−1 4	−1 3	Growth
4 1	0 0	0 0	No growth	−1 3	−1 3	Very slight growth
3 5	—	—	No growth	−0 9	−0 9	No growth
3 2	0 0	0 0	No growth	−0 4	−0 4	No growth
2 2	+0 04	0 0	No growth	+0 5	+0 6	No growth
1 8	0 0	0 0	No growth	+0 6	+0 6	No growth

In this and subsequent tables the minus sign indicates that the migration was to the anode and a plus sign indicates migration to the cathode

TABLE II

Electrophoretic Mobilities and Suspension Stability in M/50 Buffers of Varying pH

Buffer	pH of buffer	Bact typhosum S		Bact typhosum R	
		Electrophoretic mobility	Agglutination after 24 hrs	Electrophoretic mobility	Agglutination after 24 hrs
		μ/sec /volt/cm		μ/sec /volt/cm	
Acetate	5 0	0 0	None	−2 6	None
	4 7	0 0	None	−2 5	None
	4 4	0 0	None	−2 1	None
	4 1	0 0	None	−1 8	None
	3 8	0 0	None	−1 4	Partial
	3 5	0 0	None	−0 8	Complete
Phthalate	2 2	0 0	None	−0 2	Complete
	1 8	0 0	None	−0 1	Complete

ζ-potential was relatively high, and the high negative potential decreased with decrease in pH   It is also evident that the smooth form maintained a stable suspension throughout the pH range studied,

whereas the rough form maintained a stable suspension only if the pH was greater than 3 8   Thus, the bacteria were partially agglutinated when the potential was 17 5 mv , and completely agglutinated when the potential was 10 mv

A sodium acetate acetic acid buffer was made up to a concentration of 0 2 molar at pH = 5 2 (Table III)   This served as stock solution from which the concentrations of buffer indicated were obtained   In

pH of $M/50$ Buffers
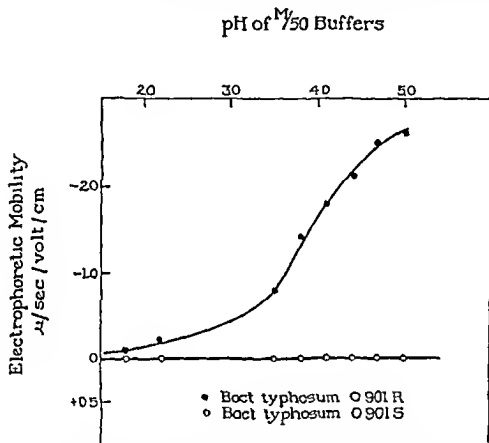
FIG 1 The electrophoretic mobility, as a function of pH of the S and R variants of *Bact typhosum* O 901, in buffer solutions of the same cation concentration

Table III are indicated the electrophoretic mobilities in μ per second per volt per centimeter with change of salt concentration, for the two forms of *Bact typhosum* O 901

The electrokinetic P D may be calculated roughly   These data show that this strain of *Bact typhosum* in the smooth form had only a very low or no electrokinetic potential, and was insensitive to precipitation or agglutination by salt, whereas, in the rough form, the electrokinetic potential was high in low concentration of salt, and decreased

with increasing concentration of salt   Furthermore, as the concentration of salt became greater, the rough form became increasingly unstable in suspension   At 20 mv (0 04 M) the rough form was almost completely precipitated, and at 6 5 mv was completely precipitated

The smooth strains described by Mudd, Nugent, and Bullock (1932) as having very low electrokinetic potentials were reexamined   Their observation was confirmed   These were strains of *Bact pullorum*, *Bact flexneri*, and *Bact coli*   Rough strains were obtained in the manner previously described   Two variants of *Bact flexneri* were obtained, R_1 and R_2 R_1 formed larger clumps as it grew in lithium

TABLE III

Electrophoretic Mobilities and Suspension Stability in Acetate Buffer of pH Approximately 5 2 and of Varying Molarity

Molarity of buffer	Bact typhosum S		Bact typhosum R	
	Electrophoretic mobility	Agglutination after 24 hrs	Electrophoretic mobility	Agglutination after 24 hrs
	μ/sec /volt/cm		μ/sec /volt/cm	
0 2	0 0	None	−0 5	Complete
0 04	−0 1	None	−1 6	Almost complete
0 02	0 0	None	−2 4	Slight
0 01	0 0	None	−3 2	Slight
0 004	+0 1	None	−3 6	Slight
0 002	+0 5	None	−3 9	Slight
0 001	−0 1	None	−3 9	Trace
Twice distilled H_2O	−1 6	None	−5 5	Trace

chloride broth, and formed a larger, flatter colony on agar   The rough variant of *Bact pullorum* may not have been a permanent form as there was a tendency for the growth in broth to become semidiffuse

Table IV indicates the change in electrophoretic mobility and in suspension stability with change in pH for the rough forms of *Bact flexneri* and *Bact pullorum*   The smooth form throughout the range studied had mobility values which very closely approached zero   These are plotted in Figs 2 and 3   The smooth forms also formed stable suspensions throughout the entire range

Occasionally a rough form either temporarily or permanently did not grow as a complete sediment in broth   One *Bact flexneri* rough

TABLE IV

Electrophoretic Mobility and Suspension Stability of Rough Strains in M/50 Buffers of Varying pH

Buffer	pH of buffer	Bact flexneri R₁		Bact flexneri R₂		Bact pullorum R	
		Electrophoretic mobility	Agglutination after 24 hrs	Electrophoretic mobility	Agglutination after 24 hrs.	Electrophoretic mobility	Agglutination after 24 hrs.
		$\mu/sec /volt/cm$		$\mu/sec /volt/cm$		$\mu/sec /volt/cm$	
Phosphate	7 27	−3 2	None	−2 9	None	−2 6	Trace
	6 3	−3 2	None	−3 0	None	−2 6	Trace
	5 95	−2 9	None	−2 8	None	−2 2	Trace
Acetate	5 9	−2 9	None	−2 6	None	−2 2	Trace
	5 6	−2 7	None	−2 7	None	−2 1	Trace
	5 3	−2 8	None	−2 6	None	−2 1	Trace
	4 7	−2 2	None	−2 2	None	−1 6	Trace
	4 4	−2 2	None	−2 3	None	−1 4	Trace
	4 1	−1 4	None	−1 8	None	−1 3	Trace
	3 8	−1 3	None	−1 5	None	−0 9	Trace
	3 5	−1 1	None	−1 2	None	−1 0	Complete
Phthalate	3 4	−1 2	Complete	−1 2	Nearly complete	−1 1	Partial
	2 8	−0 8	Complete	−0 8	Complete	−0 5	Complete
	2 2	−0 4	Complete	−0 4	Complete	+0 5	Complete

TABLE V

Electrophoretic Mobility and Suspension Stability in Acetate Buffer of Approximately pH 5 2 of Bact flexneri S and of an R Strain of Unusually High ζ Potential

Molarity of buffer	Bact. flexneri S		Bact. flexneri R	
	Electrophoretic mobility	Agglutination after 24 hrs.	Electrophoretic mobility	Agglutination after 24 hrs
	$\mu/sec /volt/cm$		$\mu/sec /volt/cm.$	
0 15	−0 1	None	−2 2	None
0 075	—	None	−2 9	None
0 05	0 0	None	−3 2	None
0 04	—	—	−3 7	None
0 015	−0 1	None	−3 7	None
0 007	—	—	−4 3	None
0 0015	−0 5	None	−5 7	None
0 0007	−0 4	None	−6 2	None
Twice distilled H₂O	−2 3	None	−4 8	None

form grew somewhat diffusely in broth   Such a situation is often considered as a tendency to revert to the smooth form (Arkwright, 1921)   The salt sensitivity of this strain was examined   The technique used was similar to that previously described   The results of the experiment are given in Table V

pH of $\frac{M}{50}$ Buffers
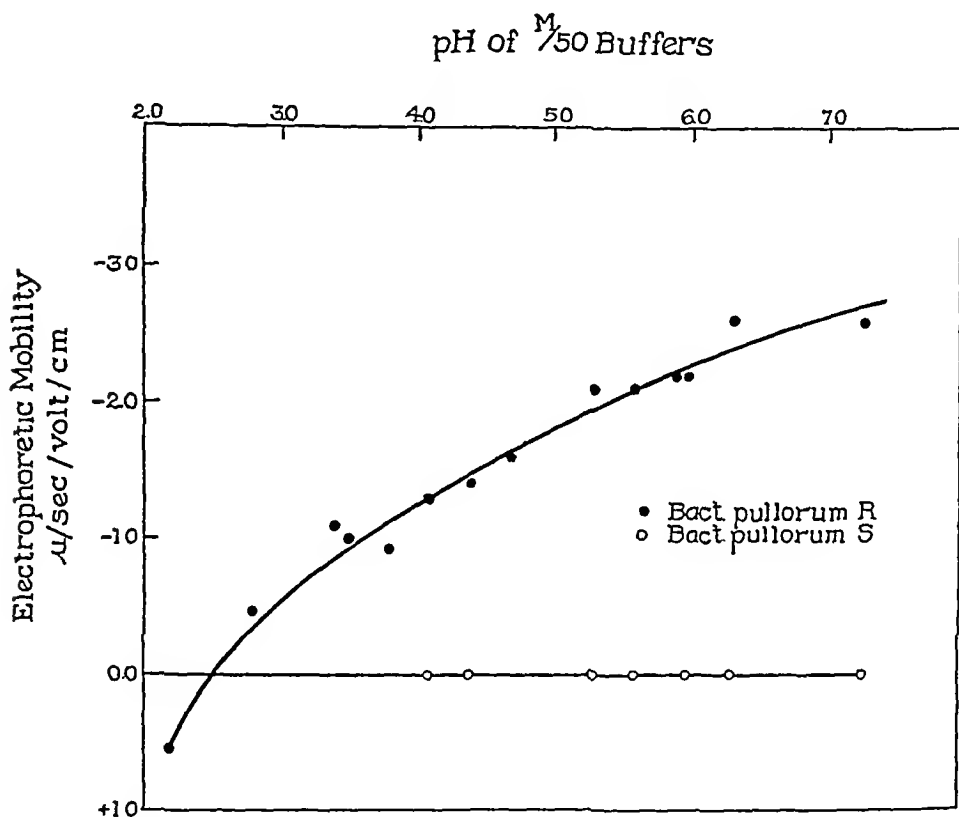
FIG 2 The electrophoretic mobility, as a function of pH, of the S and R variants of *Bact pullorum*, in buffer solutions of the same cation concentration

The potential in 0 15 M acetate buffer of approximately pH 5 2, was very high for the rough form   No agglutination took place Concomitantly the smooth form maintained its normal characteristics, namely, its suspension stability and low electrokinetic potential Ordinary nutrient broth is about 0 15 M with respect to salt concentration   This means that occasionally a rough form will have such a high electrokinetic potential that it will grow diffusely   This condi-

tion would be exaggerated if the salt concentration was for one reason or another too low   The diffuse growth of a rough strain need not necessarily imply a tendency for a rough form to revert to the smooth form, but rather it may be the result of an exceptionally high potential possessed by the rough form either temporarily or permanently [1]
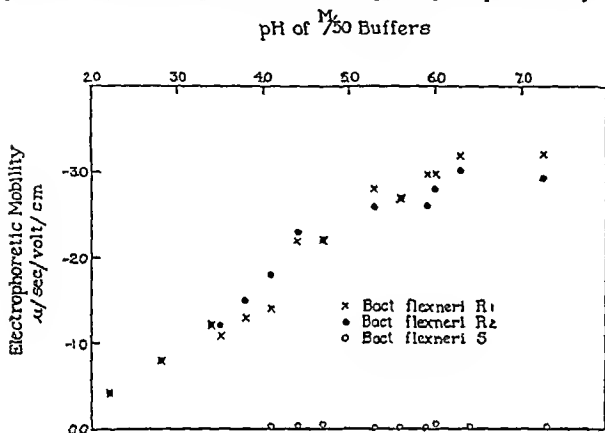
FIG 3 The electrophoretic mobility, as a function of pH, of the S and R variants of *Bact flexneri* in buffer solutions of the same cation concentration

Extensive experiments have not been performed on the rough and smooth variants of *Bact colι* and *Bact enteritidis*   However, the mobility as a function of pH was examined   In the range from pH 5 6 to 3 6 Walpole's 0 02 ɴ acetate buffers were used   Permanently non flagellated strains were not obtained   However, the develop

[1] During a single cell isolation of the rough form of *Bact typhosum* Strain O 901 and subsequent to frequent subculture diffuse growth was observed   This was thought to indicate smoothness   Rapid electrophoresis measurement showed that the strain possessed an unusually high potential   Sometime later the strain grew normally   The mechanism of a temporary unusually high potential has not been worked out. It seems clear, however, that such rough forms do not always grow as sediments

ment of flagella may be suppressed by subculture on agar containing 0 1 per cent of saturated aqueous phenol solution (Braun and Schaffer, 1919, and Feiler, 1920) With subcultures of *Bact coli* and *Bact enteritidis* grown in this manner and killed by heat, it was found that at various pH levels the electrokinetic potential of the smooth forms approximated zero, whereas the corresponding rough strains, cultured similarly, exhibited potentials quite high

These experiments indicate qualitatively similar electrokinetic properties for various intestinal bacteria Smooth non-flagellated strains have had only minimal electrokinetic potentials which remained constant regardless of variation in ion concentration or pH These strains form stable suspensions which are not agglutinated by the addition of salt or acid The change from the smooth to the rough form is accompanied by a large change in electrokinetic potential

TABLE VI

Electrophoretic Mobility in Micra per Second per Volt per Centimeter

pH	71	64	56	48	40	36	32	22
Bact coli S	−0 1	0 0	0 0	0 0	−0 1	0 0	0 0	0 0
Bact coli R	−2 7	−2 2	−2 9	−3 0	−2 9	−2 3	−2 0	−0 7

difference Rough strains have high electrokinetic potentials which do vary with change in salt concentration and with pH They do not form stable suspensions in high concentrations of salt and in solutions of low pH This inability to form stable suspensions under these conditions may be ascribed to a reduction in the electrokinetic potential below the classical critical values necessary for stability

DISCUSSION

Whether or not ζ-potentials approaching zero are in general an attribute of the non-flagellate bodies of intestinal bacteria in their smooth forms remains to be determined The occurrence of this remarkable property in four strains representative of the principal groups of intestinal bacteria is at least suggestive of generality

These smooth strains form suspensions such that their state of surface hydration is the primary stabilizing factor in their dispersion in

aqueous media over a wide range of conditions Although the
ζ potential of such suspensions is consistently low it should be pointed
out that a definite ζ potential has been observed when the suspending
medium is distilled water Whether this ζ potential is due to dissocia-
tion of components of the bacterial surface or to differential adsorption
of ions, we do not know, in either case it is suppressed in altogether an
exceptional manner by traces of electrolyte

The question of the chemical composition of these bacterial surfaces
is of course an obvious one The differences between rough and
smooth intestinal bacteria have been attributed primarily to the
properties of the carbohydrate haptenes in the surfaces of the bacterial
bodies (Heidelberger, Shwartzman, and Cohn, 1928, Turth and Land
steiner, 1929, Meyer, 1930, White, 1931) The smooth bacteria
contain soluble carbohydrate haptenes whose hydrophilic properties
appear to be responsible for the remarkable suspension stability of
these forms (White, 1928) In the corresponding rough variants
these haptenes are replaced by other carbohydrate haptenes with
much less affinity for water

In addition to the carbohydrate haptenes, the surfaces of both
smooth and rough forms contain materials extractable by absolute
alcohol-chloroform mixture at 55°C (White, 1927, 1928) These
"lipoids" are also soluble in chloroform and ether Aqueous sus
pensions are readily precipitated by electrolytes Their tendency
when incorporated in the bacterial surface appears to be to sensitize
the bacteria to precipitation by salts This tendency is masked in
the smooth forms by the predominant hydrophilic properties of the
carbohydrate haptene, in the rough forms, however, the salt sensitive-
ness becomes manifest

Evidence of the presence in the bacterial surface of a third com
ponent has been found by White in later work (White, 1932, 1933)
This is a protein antigen of very broad specificity, soluble in 95 per
cent alcohol in the presence of HCl, and precipitable by ether or salts

We are thus presented with a picture of the surface of the intestinal
bacteria as exceedingly complex In the rough non flagellate forms
the suspension stability is dominated by hydrophobic components
In the smooth non flagellate forms the suspension stability is deter-
mined by an extremely hydrophilic carbohydrate It seems likely

that the electrokinetic behavior of these smooth forms may also be dominated by this hydrophilic carbohydrate   Further discussion of this point may be found elsewhere (Mudd, 1933)

SUMMARY

The relation between electrokinetic potential and suspension stability of four strains of non-flagellate intestinal bacteria has been studied   The smooth forms have ζ-potentials which approximate zero over a wide range of pH and salt concentration, yet nevertheless form stable suspensions   The rough variants have ζ-potentials which vary with pH and electrolyte concentration in the familiar way   The rough forms have values of ζ-potential critical for their suspension stability

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A PHYSICAL-CHEMICAL DIFFERENCE IN ANTIBODIES AGAINST THE S AND R VARIANTS OF A SINGLE BACTERIAL STRAIN

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The importance and significance of chemical configuration, and its influence on antigenic specificity is well established   It has been amply demonstrated on serological grounds that a complex antigen such as the typhoid bacillus can give rise to more than one distinct antibody   Nevertheless little is known of differences in physical chemical properties of antibodies in relation to their corresponding antigens [1]   Present day evidence indicates that antibodies are probably modified globulins   The surface properties of bacteria maximally sensitized with immune sera approximate those of the sensitizing substance which is deposited upon the antigen surface from the immune serum   The properties of the sensitized surface are similar in many ways to the properties of a surface deposit of globulin

A property of the surface, susceptible of accurate measurement, is the electrokinetic potential difference (ζ potential)   From appropriate measurements of the ζ potential, an interpolated isoelectric point is obtained   The isoelectric point of a protein may be taken as an indication of the ability of the protein to react with acid or base under certain defined conditions   This in turn is a measure of the acidity or alkalinity of a protein

The surface properties of non flagellated strains of intestinal bacteria have been described (Joffe and Mudd, 1935)   The smooth form has a hydrophilic surface, with its electrophoretic mobility

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[1] In this connection, however, see Heidelberger and Kendall (1933), and Marrack (1934)

approximating zero over a wide range of pH and salt content of the medium   The rough form has a relatively hydrophobic surface, and its mobility is dependent both upon the pH and the ionic strength of the suspending medium

Mudd and Joffe (1933) found that the isoelectric points of non-flagellated smooth strains of intestinal bacteria maximally sensitized with homologous immune rabbit sera were approximately pH 5 0 Preliminary experiments heretofore unpublished seemed to indicate that the rough form, in spite of its extremely acid surface, when maximally sensitized with homologous immune serum was isoelectric at a pH higher than 5 0   The properties of these intestinal strains after sensitization might indicate that antibodies differ in their capacity to react with acid or base under the same conditions, and that the pH of the isoelectric point of the antibody would be influenced by the antigen surface upon which it may be supposed to have been formed, and subsequently upon which it was deposited (Breinl and Haurowitz, 1930, Mudd, 1933)

The present experiments were undertaken, therefore, in order to determine whether the S and R variants of a single bacterial strain could give rise to antibodies, which when deposited on the homologous bacterial surface had different isoelectric points and electrokinetic potentials

EXPERIMENTAL

Bacteria Used —In order to avoid any possible confusion which might arise from the use of mixed cultures of rough and smooth forms, single cell cultures were used   These were obtained from the stock strains of *Bact typhosum* O 901 S and O 901 R in each case by three successive single cell isolations, by Mr H E Morton, to whom I am greatly indebted   The bacteria were grown in broth of pH 7 0   18 to 20 hour cultures were then killed by incubating with 0 5 per cent HCHO solution for 24 to 48 hours, or by heat at 56° for ½ hour   When sterile the bacteria were washed in 0 85 per cent NaCl twice   Such a suspension was used both for immunization and for testing

Immunization of the Rabbits —Rabbit sera were used throughout   Each rabbit was bled from the marginal ear vein on the day previous to the first injection   The sera thus obtained were tested for agglutinins for the rough and smooth forms The rabbits were divided into two groups, one group placed on treatment with strain O 901 S and the other with strain O 901 R   The equivalent of 0 2 cc of broth culture was inoculated intravenously into each rabbit on the 1st, 3rd, and

5th days of each week. Bleedings from the ear were made on the 7th day [2]
At intervals the animals were allowed a week without infections

Sensitization by Serum —The sera used were inactivated by heat in a 56 C
water bath for 30 minutes   Only sera in which virtually no hemolysis had
occurred were used.   All dilutions of antiserum were made with 0 85 per cent
NaCl unless otherwise specified   Given volumes of bacterial suspension were
mixed with equal volumes of dilutions of antiserum   The tubes containing the
smooth bacterium as antigen were incubated for 2 hours at 37°C , and placed in
the refrigerator overnight   Agglutination was read in the morning   The tubes
containing the rough variant as antigen were incubated at 56 C   Agglutination
readings were made at intervals, until a maximum was reached before agglutina
tion of the control tubes occurred [3]   They were then refrigerated

After refrigeration   all tubes were centrifuged until virtually complete sedi
mentation had taken place   The supernatant fluids were decanted and the sedi
ments shaken up in 3 ml each of 0 85 per cent NaCl   All tubes were again centri
fuged the supernatants decanted, and the sediment shaken up

Electrophoresis and Estimation of Isoelectric Points —Electrophoresis measure
ments were made in the manner described in the previous paper   0 02 ℳ sodium
acetate acetic acid buffers of constant ionic strength were made up so that succes
sive members of the series differed by 0 3 pH unit   Two successive buffers were
found in one of which the particles migrated to the anode and in the other to the
cathode   The isoelectric point was then estimated from the relative mean veloci
ties at the two pH values   pH values were checked by a quinhydrone electrode,
using as a reference standard a pH of 1 08 for 0 1 N HCl   When necessary Sør
ensen's phosphate and Clark and Lubs phthalate buffer mixtures were used in
suitable concentration

Agglutination tests and isoelectric point determinations were made on the sera
obtained each week   Each serum was caused to react with its homologous antigen,
and where possible with the heterologous antigen

Glassware —All glassware was chemically cleaned with sulfuric acid potassium
dichromate solution and washed in water and distilled water very thoroughly

RESULTS

The essential results are shown in Fig 1 and in Table I   The
smooth form of 0 901 S had little or no ʃ potential in any buffer studied

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[2] Thanks are due Mr David Lackman for technical assistance

[3] The temperatures used for accelerating flocculation were used for convenience
Rough   agglutinations can be most easily read at 56° in the manner described.
Within the period measured the difference in temperature between 56° and 37°
probably has very little effect on the pH of the isoelectric point   and when the
smooth variant was sensitized at 56 (see p 624) the isoelectric points obtained
were similar to those obtained when the same sera were used to sensitize at 37

Duration of Immunization in Days

Fig 1

618

Sensitization with immune serum conferred upon it a definite mobility, from which an isoelectric point was determined   This isoelectric point, then, an over all measurement, was due to a deposition of antibody on the bacterial surface, and was dependent upon the antibody alone   In the case of the rough form, whose surface was very acid,

TABLE I

The progressive shift to the alkaline side of the pH of the isoelectric points of bacteria maximally sensitized with homologous immune rabbit serum obtained at weekly intervals during continued immunization

Homologous antigen	Serum No	Time of immunization in days												
		0	7	14	21	28	35	42	49	56	63	70	77	91
		pH of isoelectric point												
O 901 S	201		4 8		5 15	5 25	5 2	5 3		5 3				5 4
O 901 S	202		4 95	5 0	5 2	5 3	5 4			5 25				
O 901 S	203		4 8	4 8	5 3	5 2	5 2	5 3		5 25				
O 901 S	205		4 75	4 95	5 1	5 2	5 2	5 3		5 3				5 5
O 901 S	207		4 8	4 9	5 3	5 2	5 3	5 3		5 25				5 5
Average			4 82	4 91	5 21	5 21	5 24	5 32		5 28				5 47
O 901 R	391	5 15	4 95	5 05	5 3									
O 901 R	392	5 0	4 85	5 15	5 25	5 25	5 45	5 6		5 55	5 5	5 9	5 85	
O 901 R	394	4 95	5 05	5 1										
O 901 R	396	5 1	4 9	5 1	5 1	5 25	5 35	5 6		5 65	5 7	5 9	5 75	
O 901 R	397		4 95	5 1	5 1	5 15	5 5	5 6		5 7	5 8	5 8	5 8	
O 901 R	316	5 05	5 2	5 05	5 1	5 3	5 3	5 5	5 4					
O 901 R	317	5 0	4 9	5 2	5 05	5 3	5 4	5 5	5 4					
O 901 R	283	5 0	4 9	5 2	5 2		5 6	5 8						
Average		5 05	4 96	5 1	5 15	5 21	5 43	5 57		5 63	5 67	5 87	5 8	

the surface deposit of antibody decreased the negative ζ potential, but only when a value was reached which did not change upon the

Fig 1   Abscissae   The duration of immunization in days   Ordinates   The upper blocks indicate the pH of the isoelectric points for the maximally sensitized bacteria

The lower blocks indicate the final agglutination titers of the sera   The scale divisions represent the following successive serum dilutions   1 2 4, 16 64 256 1024 4096 etc   Thus scale division 5 indicates an end titer at a serum dilution of 1 256

addition of more immune serum could the isoelectric point be considered as that of the surface deposit

When rabbits were immunized with the smooth form, the agglutination titer for the smooth form of the sera obtained at weekly intervals rose rapidly, reaching a maximum at or before the 28th day The isoelectric point of the bacteria, maximally sensitized, about pH 4 8 on the 7th day, rose gradually beyond the 56th day It reached a value of pH 5 3 at that time

When rabbits were immunized with the rough form, the homologous agglutination titer of the serum obtained at weekly intervals rose more slowly at first, it reached a maximum at about the same time as the titer for the sera against the smooth form The isoelectric point of the rough form sensitized with normal serum was about pH 5 0 to 5 1 After the 1st week of immunization there was a slight drop, after which there was a steady, rapid shift of isoelectric points to the alkaline side Until the 35th day there was no great difference between the rough variant and the smooth variant maximally sensitized with homologous antisera After the 35th day the maximally sensitized rough form had a distinctly more alkaline isoelectric point, it reached a value of pH 5 6 on the 56th day, and the exceedingly high value pH of 5 9 on the 70th day

Thus two antisera prepared from the same complex antigen, may have the same agglutination titer, whereas the surface properties of the antibodies, when deposited upon the homologous bacterial surfaces, may be very different

The smooth form and the rough form cross-reacted to about the same degree Maximum agglutination titer in cross-reaction was usually at a serum dilution of 1 256 Occasionally agglutination at a dilution of 1 1024 was observed The isoelectric point of the smooth form maximally sensitized with rough serum rose from a value of pH 4 8 on the 14th day to about pH 5 1 on the 56th day and slightly more than pH 5 2 on the 70th day The rough form maximally sensitized with smooth antiserum had a rather steady value of pH 5 0 until the 56th day This may have been due to incomplete saturation with antibody of acid groups on the surface of the bacterium

In Fig 2 are plotted the isoelectric points of the rough and smooth forms sensitized with increasing concentrations of the homologous

immune serum Curves I and II are sensitized rough forms and Curves III and IV are sensitized smooth forms There was a marked

Dilution of Homologous Antiserum
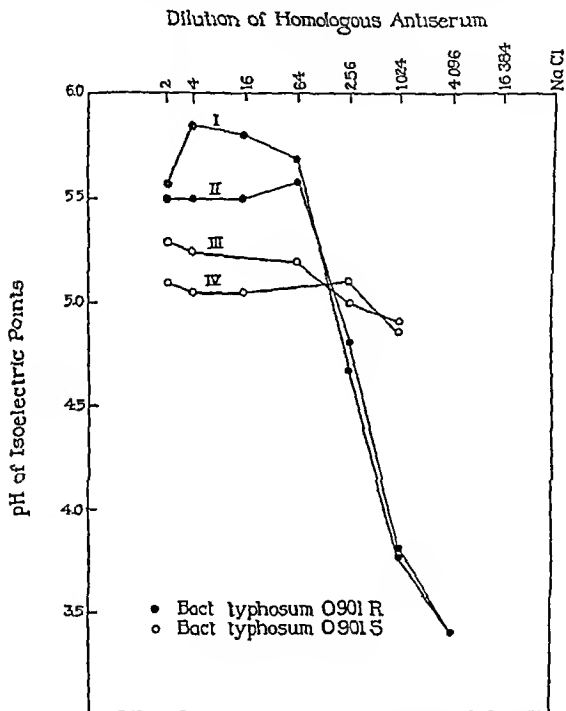
Fig 2 The R and S variants of *Bact typhosum* O 901 were sensitized with increasing concentrations of homologous immune sera Curve I, Serum 392, drawn 70th day of immunization Curve II, Serum 397 drawn 63rd day, Curve III, Serum 201, drawn 91st day Curve IV Serum 203, drawn 63rd day

difference in the general shape of the curves of the sensitized rough and smooth forms The flatness of the curve of the smooth form would

seem to indicate that the major factor in determining the isoelectric point is the surface deposit of antibody   On the other hand, the steep slope of the curve of the sensitized rough form at higher dilutions of serum would seem to indicate the definite influence, in this region of the curve at least, of the underlying electronegative bacterial surface
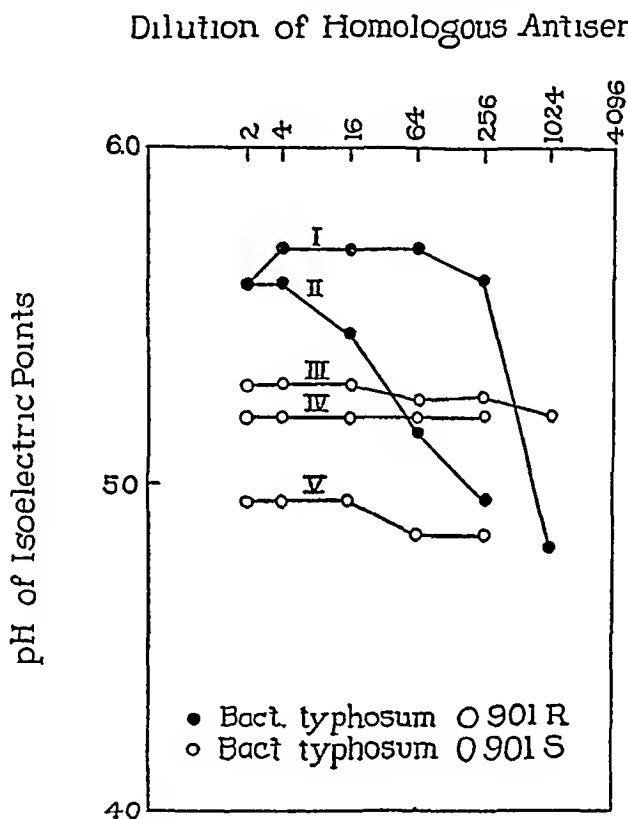
FIG 3   The R and S variants were sensitized with increasing concentrations of homologous immune sera   Curve I, Serum 62, hyperimmune, Curve II, Serum 392, drawn 42nd day, Curve III, Serum 499, hyperimmune, Curve IV, Serum 7, hyperimmune, Curve V, Serum 7, drawn approximately the 21st day

The difference between the isoelectric points of maximally sensitized rough and smooth forms is of still greater significance when one considers that the more acid surface of the rough variants, when maximally sensitized with homologous antiserum, is more alkaline than the inert surface of the smooth form sensitized with its homologous antiserum.

Fig 3 is supplementary to Fig 2 This shows data obtained from the sera of rabbits immunized with 0 901 S and 0 901 R (not single cell

pH of $^M/_{50}$ Buffers
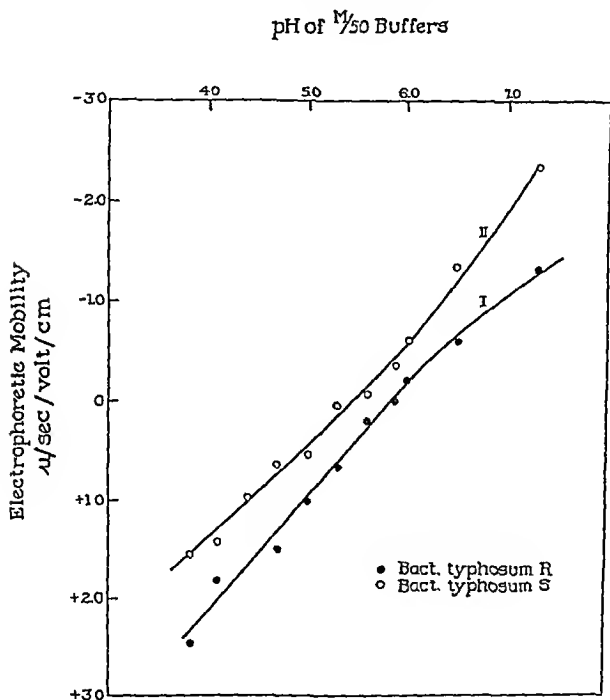
FIG 4 The electrophoretic mobilities as a function of pH of the S and R variants of *Bact typhosum* O 901 maximally sensitized with their homologous immune sera The measurements were made in buffer solutions of approximately the same cation concentration

strains), over a long period of time, previous to the beginning of the control experiments herein reported When obtained these data were

very confusing In the light of the present experiments, however, the data seem to be intelligible

In Fig 4 are plotted the electrophoretic mobilities at decreasing values of pH of the rough and smooth forms maximally sensitized with homologous antisera The bacteria were sensitized in individual tubes, and incubated at 56°C for 1 hour (at which time they were agglutinated) They were then refrigerated, centrifuged, washed, and resuspended as homogeneously as possible in M/50 buffer solutions They were then incubated at 56°C for 2 hours and refrigerated overnight

Two anti-smooth sera and two anti-rough sera were used They were chosen so that they were drawn from animals which had received approximately the same amount of vaccine treatment In the figure drawn the sera were obtained at the end of the immunizing period The isoelectric points differ by 0 4 to 0 5 pH and the curves of mobility differ throughout the range of pH studied

SUMMARY

1 Rabbits were immunized with *Bact typhosum* 0 901 S and 0 901 R, over a long period Homologous and heterologous strains were sensitized with sera obtained from weekly bleedings Agglutination titer was recorded, and the isoelectric points of the bacteria maximally sensitized were determined

2 0 901 S maximally sensitized with homologous immune serum had isoelectric points which became more alkaline as immunization progressed, covering a range of pH 4 8 to 5 5

3 Strain 0 901 R maximally sensitized with homologous immune serum had isoelectric points which became more alkaline as immunization progressed, covering the range of pH 5 0 to 5 9

4 Both 0 901 S and 0 901 R maximally sensitized with heterologous serum had isoelectric points lower than when sensitized with homologous serum

5 The isoelectric points of both forms sensitized with increasing concentrations of homologous immune serum were determined Increasing concentrations of homologous immune serum shifted the isoelectric point of 0 901 R from less than 2 2 for the unsensitized bacteria progressively to the alkaline side until the maximum values

previously mentioned were reached Increasing concentrations of homologous immune serum conferred upon 0 901 S isoelectric points which became only slightly more alkaline in maximal sensitization

6 The electrophoretic mobilities of 0 901 S and 0 901 R, in each case maximally sensitized with homologous hyperimmune serum, were found to differ significantly over the whole range of pH studied

CONCLUSIONS

1 Antibodies to the rough and smooth variants of *Bact typhosum* 0 901, when studied as deposits on the maximally sensitized bacterial surfaces, differed significantly in isoelectric points and in ζ potentials

2 With increasing time of immunization of rabbits the isoelectric points of the sensitizing antibody deposits shifted progressively to the alkaline side, agglutination titers did not change concurrently   Thus two sera may have the same agglutination titers, but may form, on the homologous bacterial strains, sensitizing surface deposits whose iso electric points differ by 0 5 to 0 8 pH unit

3 Rabbits immunized with single cell strains of 0 901 S gradually developed antibodies for 0 901 R and *vice versa*

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THE THEORY OF DIFFUSION IN CELL MODELS

II Solution of the Steady State for Three Diffusing Substances

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I

INTRODUCTION

Osterhout and Stanley,[1] and, more recently, these authors together with Kamerling [2] have reported measurements on the simultaneous diffusion of one or more salts and water in cell models   The diffusion takes place between two aqueous layers, designated by A and C, which are separated by a non aqueous layer, B The composition of the aqueous phase A is held constant and carbon dioxide is passed through the phase C The salts present in A are metallic salts of the non aqueous material in B, this material being a weak acid   These salts diffuse through B, in which they are appreciably soluble, into C where they are converted almost entirely into the bicarbonates and are thereby "trapped" in this phase owing to the insolubility of the bicarbonates in the non aqueous material of B

The salt diffusion is accompanied by changes in the osmotic activity of the water in the various phases and this material consequently diffuses simultaneously with the salts   The direction in which water first diffuses depends upon the initial conditions of the experiment but after a short interval of time the solute concentration in C becomes greater than in A and water then diffuses from A, through B, into C at a rate which increases with the time   The rate of diffusion of

[1] Osterhout W J V and Stanley, W M , *J Gen Physiol*, 1931–32, 15, 667

[2] Osterhout W J V , Kamerling S E and Stanley, W M , *J Gen Physiol*, 1933–34 17, 445

[3] Osterhout W J V , Kamerling S E and Stanley W M , *J Gen Physiol*, 1933–34, 17, 469

salt from A to C simultaneously decreases so that a steady state is approached in which the entrance of salts and water into C is in such a ratio that the concentrations prevailing there are not altered   Consequently, in the steady state the concentrations of the various substances in phase C remain constant although the volume of this phase increases linearly with the time

In the first paper of this series[4] a theory was presented which satisfactorily accounted for the entire course of the diffusion in a cell model involving the movement of only one salt and water   It is the purpose of this paper to extend the theory to the simultaneous diffusion of two salts and water and to solve the resulting system of three differential equations for the steady state   The complete solution for this system of equations, which would thus permit the computation of the various time curves, has not yet been obtained   The solution for the steady state, however, is of special interest because it indicates the conditions under which the preferential accumulation of an electrolyte can occur Moreover, it has also been shown[5] that the volume change of phase C with the time when the system is in a steady state is somewhat analogous to the growth of a living cell

II

Formulation of the Equations

Since the results of this analysis are to be compared with a variety of salt pairs it is desirable to keep the various symbols as general as possible   The same nomenclature as used in the first paper will be retained, with the following generalizations which, it is hoped, will not prove confusing   The subscripts 1 and i refer to the guaiacolate of a cation of species 1 and the bicarbonate of the same species, respectively   The subscripts 2 and ii refer to the corresponding salts of a second species of cation, while 0 refers to water

Moreover, since no quantitative comparison between theory and experiment is possible, as will be shown in Section IV, it seems desir-

[4] Longsworth, L G , *J Gen Physiol*, 1933-34, **17**, 211

[5] Longsworth, L G , The theory of diffusion in cell models and volume changes analogous to growth, in Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, Long Island Biological Association, 1934, **2**, 218

able to simplify the expressions somewhat by means of the following conventions

1 The variation of the partition coefficients at the two phase boundaries will be ignored, so that $S^a = S^b{}_i$

2 The aqueous solutions will be considered ideal so that $\imath^A = \imath^C = 2$

3 The effect of the solubility of CO_2 upon the water activity in phase C will be ignored in comparison with the effect of the salts

The foregoing simplifications do not alter the form of the equations but merely serve to focus attention upon the relation of the most important parameters to the characteristics of the steady state

In strict analogy to equations (6 2) and (6 3) of the first paper of this series the following equations may be written

$$\frac{dn_0^C}{dt} = \frac{AD_0}{V_{HG}\Delta x}\left(S_0 N_0^A - S_0 N_0^C\right) \simeq \frac{2 AD_0 S_0}{V_{HG}\Delta x}\left(-N_1^A - N_2^A + \frac{n_I^C}{n_0^C} + \frac{n_{II}^C}{n_0^C}\right) \quad (2\ 1)$$

$$\frac{dn_I^C}{dt} = \frac{AD_1}{V_{HG}\Delta x}\left(S_1 N_1^A - S_1 N_1^C\right) \simeq \frac{AD_1 S_1}{V_{HG} K\Delta x}\left(K N_1^A - \frac{n_I^C}{n_0^C}\right) \quad (2\ 2)$$

$$\frac{dn_{II}^C}{dt} = \frac{AD_2}{V_{HG}\Delta x}\left(S_2 N_2^A - S_2 N_2^C\right) \simeq \frac{AD_2 S_2}{V_{HG} K\Delta x}\left(K N_2^A - \frac{n_{II}^C}{n_0^C}\right) \quad (2\ 3)$$

It should be noted that the same constant is used for the two equilibria

$$Salt_1 + H_2CO_3 = Salt_I + HG$$
$$Salt_2 + H_2CO_3 = Salt_{II} + HG$$
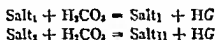
This is permissible since both equilibria reduce to the single ionic process,

$$G^- + H\,CO_3 = HCO_3{}^- + HG$$

Equations (2 2) and (2 3) have been formulated in a manner that is consistent with the definition of the partition coefficient as given in the first paper, namely

$$S_i = \frac{N_i^B}{N_i^A} \quad (2\ 4)$$

Moreover, the experimental values for S_i, as reported by Osterhout and associates,[3] are also consistent with this definition and were measured

in the presence of a second alkali guaiacolate in A so that the increase in the "effective concentration" of the first salt, due to the common ion of the second salt, is reflected in an increased value for the partition coefficient   Thus $S_{KG} = 0\,95$ when only KG is present in A and $N_{KG}^A = 0\,00036$, but the value of this partition coefficient is increased to $1\,33$ when NaG is also present in A at the same concentration   The latter value for S_{KG} must be used in the computations of this paper for systems involving sodium and potassium   If KG and NaG were completely ionized in A and the ions were perfect solutes, the "effective concentration" of KG, for example, could be identified with the ionic product, $(N_{K^+}^A)\,(N_{G^-}^A)$ [3]   Moreover, if KG was a perfect *non-electro-lytic* solute in B, a partition coefficient defined by the relation

$$\sigma_{KG} = \frac{N_{KG}^B}{(N_{K^+}^A)\,(N_{G^-}^A)} \tag{2 5}$$

would be a constant independent of the concentration   Actually, however, the salts are neither perfect electrolytes in A nor perfect non-electrolytes in B Consequently the partition coefficient defined by equation (2 5) is but slightly less dependent upon the concentration than that defined by (2 4)   Shedlovsky and Uhlig[6] have been able to account for the variation of the partition coefficient with the concentration but were unable to express S as an explicit function of the latter   Neither their treatment nor the coefficient defined by equation (2 5) can be readily adapted to the present problem and, as explained above, the definition given by (2 4) will be used throughout this paper

It should also be noted that in equations (2 1), (2 2), and (2 3) the diffusion coefficient D and partition coefficient S for a given substance always occur as the product   Thus a salt for which D is large and S is small, relatively, will behave kinetically as one for which D is small and S large   The parameters which are essential to the theory are therefore the products D_iS_i, which will henceforth be written simply P_i, the initial concentrations N_i^A, the factor $\dfrac{A}{V_{HG}\,\Delta v}$, and the equilibrium constant K The dependent variables are n_o^C, n_I^C, and n_{II}^C, and

⁶ Shedlovsky, T , and Uhlig, H H , *J Gen Physiol*, 1933–34, **17**, 563

t is the independent variable In subsequent expressions the superscript denoting phase C may be dropped without ambiguity

III

Solution for the Steady State

Differentiation of the expressions

$$N_I = \frac{n_I}{n_o}$$

$$N_{II} = \frac{n_{II}}{n_o}$$

with respect to the time gives the relations

$$n_o \frac{dN_I}{dt} + N_I \frac{dn_o}{dt} = \frac{dn_I}{dt} \tag{3 1}$$

$$n_o \frac{dN_{II}}{dt} + N_{II} \frac{dn_o}{dt} = \frac{dn_{II}}{dt} \tag{3 2}$$

In the steady state, however,

$$\frac{dN_I}{dt} = \frac{dN_{II}}{dt} = 0$$

and

$$N_I = N_{Is} \qquad N_{II} = N_{IIs}$$

so that equations (3 1) and (3 2) become

$$N_{Is} \left(\overline{\frac{dn_o}{dt}}\right)_s = \left(\frac{dn_I}{dt}\right)_s \tag{3.3}$$

$$N_{IIs} \left(\frac{dn_o}{dt}\right)_s = \left(\frac{dn_{II}}{dt}\right)_s \tag{3 4}$$

Elimination of $\left(\frac{dn_o}{dt}\right)_s$ between these two equations gives

$$N_{Is} \left(\frac{dn_{II}}{dt}\right)_s = N_{IIs} \left(\frac{dn_I}{dt}\right)_s$$

or, with reference to equations (2 2) and (2 3),

$$P_2 N_{I_s} \left(K N_2^A - N_{II_s} \right) = P_1 N_{II_s} \left(K N_1^A - N_{I_s} \right) \tag{3 5}$$

If R_s denotes the ratio N_{I_s}/N_{II_s} and $P_{1/2}$ the ratio P_1/P_2, this equation may be rewritten as

$$N_{I_s} = K \frac{P_{1/2} N_1^A - R_s N_2^A}{P_{1/2} - 1} \tag{3 6}$$

or

$$N_{II_s} = \frac{K}{R_s} \frac{P_{1/2} N_1^A - R_s N_2^A}{P_{1/2} - 1} \tag{3 7}$$

Equation (3 5) represents a simple relationship between the concentrations of the two salts in the steady state such that if one concentration is known the other may be easily computed   The computation of one of the steady state concentrations may be accomplished as follows

If the expressions for $\left(\dfrac{dn_0}{dt}\right)_s$ and $\left(\dfrac{dn_I}{dt}\right)_s$, as given by equations (2 1) and (2 2), are substituted in equation (3 3) there results

$$2 P_0 K N_{I_s} \left(-N_1^A - N_2^A + N_{I_s} + N_{II_s} \right) = P_1 \left(K N_1^A - N_{I_s} \right) \tag{3 8}$$

Elimination of N_{II_s} between this expression and equation (3 5) gives, after rationalization,

$$N_{I_s}^3 - \left[N_1^A + N_2^A + (P_{1/2} N_1^A + N_2^A) \frac{K}{P_{1/2} - 1} - \frac{P_{1/0}}{2K} \right] N_{I_s}^2 + \left[\left(N_1^A + N_2^A - \frac{P_{1/0}}{2K} \right) \right.$$

$$\left. \frac{P_{1/\cdot} K N_1^A}{P_{1/2} - 1} - \frac{1}{2} P_{1/0} N_1^A \right] N_{I_s} + \frac{P_{1/0} P_{1/2}}{P_{1/2} - 1} \frac{K \overline{N_1^A}^2}{2} = 0 \tag{3 9}$$

and an entirely analogous expression may be derived for N_{II_s}
     Numerical computation is simpler, however, with the equation involving only the ratio, R_s This equation may be obtained by substituting the values for N_{I_s}, and N_{II_s}, as given by equations (3 6) and (3 7), into equation (3 8)   The result, after rationalization, is

$$R_s^3 - \left[2P_{1/2}\frac{N_1^A}{N_2^A} + \left(\frac{P_{1/0}}{2K} - N_1^A - N_2^A\right)\frac{P_{1/2}-1}{KN_2^A} - 1 \right]R_s^2 + \left[\left(P_{1/2}\frac{N_1^A}{N_2^A}\right)^2 \right.$$

$$- 2P_{1/2}\frac{N_1^A}{N_2^A} + \left(\frac{P_{1/0}N_1^A}{2(KN_2^A)^2} - \frac{N_1^A(N_1^A + N_2^A)}{K(N_2^A)^2}P_{1/2}\right)(P_{1/2}-1) \right]R_s$$

$$+ \left(P_{1/2}\frac{N_1^A}{N_2^A}\right)^2 = 0 \tag{3 10}$$

Substitution of this value of R_s in equations (3 6) and (3 7) then gives N_I and N_{II} directly

Thus a computation of the concentrations in the steady state, by either equation (3 9) or (3 10), involves the solution of ordinary cubic equations   In these expressions the ratios $\frac{P_1}{P_2}$ and $\frac{P_1}{P_0}$ have been written as $P_{1/2}$ and $P_{1/0}$, respectively, and it is evident that the concentrations in the steady state are functions of these ratios only and do not depend upon the individual values   It may be noted that the term $\frac{A}{V_{HG}a\Delta x}$ is not present in equations (3 1) to (3 10) and hence has no effect on the concentrations in the steady state although the value of this factor will affect the time curve and the "growth" or volume increase of phase C after the steady state has been approached   The parameters which determine N_I, N_{II}, and R, are therefore $P_{1/2}$, $P_{1/0}$, K, N_1^A, and N_2^A

<div style="text-align:center">IV</div>

<div style="text-align:center">Comparison of Theory with Experiment</div>

Osterhout, Kamerling, and Stanley[2] [3] have studied experimentally the simultaneous diffusion of several pairs of alkali guaiacolates in the manner described in the introduction   However, in none of the series of experiments, with one exception, did they allow the diffusion to proceed until the steady state was definitely approached   Consequently any values for R_s which might be computed from equation (3 10) would not be comparable with the experimental results obtained by these authors   However, the ratio, R, of the salt concentrations in phase C which they report might be expected to lie between the value,

R_0, at zero time and R_s, the value in the steady state   It seems worth while, therefore, to compute values of R_s

The experimental data which are essential for the following computations and comparisons are recorded in Table I   The results for a given salt pair and for a given concentration in phase A are grouped together   In the original presentation of the experimental results volume concentrations were used   These have been converted to mole fractions with the factor $\dfrac{1}{55\ 5}$ for the aqueous solutions and the factor $\dfrac{1}{14\ 4}$ for the non-aqueous phase   The first factor is the reciprocal of the number of moles of water in 1 liter of the aqueous phase, the number of moles of guaiacol in this phase being negligible, while 14 4 is the sum of the number of moles of guaiacol, 8 4, and the number of moles of water, 6 0, in 1 liter of the non-aqueous phase   Partition coefficients on a volume basis must be multiplied by $\dfrac{55\ 5}{14\ 4}$ in order to convert them to the mole fraction basis   The subscript 1 has been assigned to the salt with the larger partition coefficient so that the values of $S_{1/2}$, which are recorded in Column 7 of Table I, are always greater than unity N_I (Column 5) is the concentration of the bicarbonate of cation 1 in phase C after the experiment had been in progress for the number of days recorded in the fourth column R (Column 6) is the value of N_I/N_{II} when N_I has the value given in the preceding column R is a rather complex function of N_I and, since the latter shows considerable variation among the experiments of a given group, the average value of R which has been computed for each group in Table II has only qualitative significance

Before numerical computations with equation (3 10) can be made, values must be assigned to the various parameters N_1^A and N_2^A, which were equal to each other in every instance, are known from the conditions of the experiment, and S_1 and S_2 have been measured for all of the salt pairs which were studied   Moreover, it has been observed[3] that all of the alkali guaiacolates have essentially the same diffusion coefficients in guaiacol so that $P_{1/2}$ becomes simply $S_{1/2}$ The constants $P_{1/0}$ and K have not been measured independently but sufficient data have been reported for one diffusion experiment

(Experiment 66), which was allowed to approximate to the steady state, to permit the evaluation of these constants   Thus in Experiment 66, for which $S_{1/2} = P_{1/2} = 2\ 14$ and $N_1^A = N_2^A = 0\ 0009$, N_{1s}

TABLE I

Summary of Results of Osterhout Kamerling and Stanley

1	2	3	4	5	6	7	8
Salt₁ Salt₂	$N_1^A = N_2^A$	Experi ment No.	Time	N_s	R	$S_{1/2}$	S_2
			days				
Cs–Na	0 00036	114a	4	0 00099	4 76	9 6	4 90
		b	9	0 00173	4 20		
					Av 4 5		
Rb–Na	0 00036	113a	4	0 00106	2 34	3 5	2 39
		b	7	0 00197	2 94		
					Av 2 6		
K–Li	0 00036	108a	8	0 00320	3 07	5 2	1 57
		b	8	0 00450	2 84		
		c	7	0 00147	3 12		
		d	9	0 00153	3 72		
					Av 3 1		
K–Na	0 00036	109a	10	0 00185	2 45	2 12	1 33
		b	8	0 00175	2 36		
		c	11	0 00193	2 18		
					Av 2 3		
K–Na Steady state	0 00090	66	33	0 01317	1 62	2 14	2 36
		80	4		2 7		
		81	4		2 7		
		111	50		2 0		
					Av 2 2		
K–Na	0 0018	58	9		1 4	2 3	4 12
		63	12		1 5		
					Av 1 4₅		

$= 0\ 01317$, and $R = 1\ 62$ Substitution of these values in equation (3 6) gives a value for $K = 32$ Further substitution of these values, including that for Λ which has just been obtained, in equation (3 10) gives a value of $P_{1/0} = 1\ 045$ These values for Λ and $P_{1/0}$ compare

favorably with the corresponding values, 21 and 1 02, respectively, from the first paper of this series No exact comparison is possible, however, since the values which have just been computed are affected by the simplifying assumptions which were made in Section II In Experiment 66 the subscripts 1 and 2 refer to potassium and sodium respectively, and since $S_1 = 2\,36$, $\dfrac{P_{1/0}}{S_1} = \dfrac{D_1}{D_0 S_0} = 0\,443$ For the other salt pairs, $P_{1/0}$ may be obtained by taking $0\,443\,S_1$ Thus for the cesium-sodium experiments, $S_1 = 4\,90$ and $P_{1/0} = 2\,171$, it being recalled that $D_1 = D_2$ although $D_1 \neq D_0$

In the numerical solution of equation (3 10) it has been found convenient to use the following standard procedure Rewriting this equation as

$$R_s^3 - A R_s^2 + B R_s - C = 0 \tag{4 1}$$

the substitution

$$R_s = ky + \frac{A}{3} \tag{4 2}$$

in which

$$k^3 = -\left(\frac{A}{3}\right)^3 + \frac{AB}{6} - \frac{C}{2} \tag{4 3}$$

reduces equation (4 1) to

$$y^3 + 2 = 3\,py \tag{4 4}$$

in which

$$p = \frac{1}{3k^2}\left(\frac{A^2}{3} - B\right) \tag{4 5}$$

In equation (4 4), y is a trivalent function of the single parameter $3\,p$ and can be tabulated in a given region as accurately as required The tables in Jahnke-Emde[7] were found to be satisfactory

As an example of the use of these tables, R_s for Experiment 66 may be computed Since $P_{1/2} = 2\,14$, $P_{1/0} = 1\,045$, $K = 32$, and N_1^A

[7] Jahnke, E, and Emde, F, Funktionentafeln, Leipsic, B G Teubner, 2nd edition, 1933, p 27

$= N_2^4 = 0\ 00090$, the coefficients of equation (4 1) become $A = 3\ 855$, $B = 0\ 7933$, and $C = -4\ 58$ Substitution of these values in equation (4 3) gives a value for $k = 0\ 8784$ and hence in equation (4 5), $3\ p = 5\ 39$ The three roots in the tables[7] corresponding to this value of p are $y_1 = 2\ 107$, $y_- = 0\ 3813$, and $y_3 = -2\ 489$ The smallest positive root of equation (3 10) is the only physically possible one and in the present example this is given by y_2, so that

$$R = ky_4 + \frac{A}{3} = 1\ 62$$

Substitution of this value for R, in equation (3 6) gives a value for $N_{1_e} = 0\ 01317$, these values for R and N_1 being identical with the

TABLE II

Computation of R_s and N_{L_s} for $K = 32$ and $P_{1/0} = 0\ 443\ S_1$

		Cs–Na	Rb–Na	K–Li	K–Na	K–Na	K–Na
1	Salt$_1$–salt$_2$						
2	$N_1^2 = N_2$	0 00036	0 00036	0 00036	0 00036	0 00090	0 0018
3	$S_{1/2} = P_{1/2}$	9 6	3 5	5 2	2 12	2 14	2 3
4	S_1	4 90	2 39	1 57	1 33	2 36	4 12
5	$P_{1/0}$	2 171	1 059	0 695	0 589	1 045	1 825
6	N_{L_s}-calc	0 00872	0 00715	0 00630	0 00587	0 01317	0 0253
7	R_s-calc	3 09	1 95	2 90	1 55	1 62	1 73
8	R_s avg (Table I)	4 5	2 6	3 1	2 3	2 2	1 4$_5$
9	$R_0 = S_{1/2}$	9 6	3 5	5 2	2 12	2 14	2 3

experimental values which were used in the evaluation of K and $P_{1/0}$, thereby furnishing a check upon the numerical solution of equation (3 10)

The values of R and N_{1_e} for the various series listed in Table I were computed as in the preceding example and are recorded in Rows 6 and 7 of Table II The values of the parameters which were used in these computations are recorded in the first five rows of the table The average values of R from Table I are recorded in the eighth row of Table II, while in the last row the values of $S_{1/2}$ are repeated, since $S_{1/2}$ is also the value of R at zero time or R_0[6] For the first three salt pairs in the table the value of R ₛ ₑᵣₐ ₓ ₑ is intermediate between R

and R_0 as would be expected   This is also true for the potassium-sodium series if an average of

$$R_{\text{average}} = \frac{1}{3}(2\,2 + 2\,3 + 1\,4_5) = 2\,0$$

for all three series is taken   However, when considered individually, the potassium-sodium series show deviations from the expected behavior, although *these deviations are of the same order of magnitude* as the deviations of the individual values from the mean

A comparison of N_{I_s} in a given column of Table II with the individual values of N_I for the corresponding series in Table I indicates that the experiments were usually interrupted long before the steady state had been approached   It may be significant, however, that in Experiment 108 b, which was allowed to approach the steady state much more closely than any other experiment, the observed value for $R = 2\,84$ is in good agreement with the computed value for $R_s = 2\,90$

Osterhout, Kamerling, and Stanley have also computed their results so as to be able to express the accumulation of a given alkali salt in C relative to cesium   Following the procedure of these authors the relative accumulation, expressed as per cent of the simultaneous value for cesium, has been plotted in Fig 1 against the partition coefficient of the corresponding alkali guaiacolate, the experimental points being indicated by circles   *In the notation of the present paper this is a plot of* $\frac{100}{R}$ *versus* S_2 for a series of salt pairs in which the subscript 1 (or 1) refers to cesium   The author has computed values of R_s for such a series by means of equation (3 10), retaining the values $K = 32$ and $P_{1/0} = 0\,443\,S_1$, and the results, plotted as $\frac{100}{R_s}$, are indicated by the full curve in Fig 1   This curve represents the relative accumulation which would have occurred if the experiments had approximated to the steady state   The straight dashed line in Fig 1 is a plot of $\frac{100}{R_0}\left(=\frac{100}{S_{1/2}}\right)$ and represents the relative accumulation which occurs in the early stages of the experiment   Without exception the experimental points lie within the area bounded by the full and dashed lines, as required by the theory
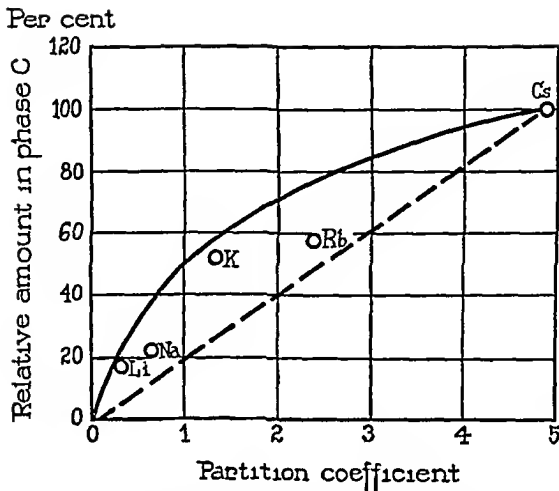
FIG 1 A comparison of theory with experiment for the accumulation relative to cesium, of a second alkali metal bicarbonate in phase C of the model The ordinates indicate the concentration of the second salt in C expressed as per cent of the simultaneous value for cesium If the ordinate for the second salt is less than 100 preferential accumulation of cesium is indicated since the guaiacolates of both metals are present in phase A at equal concentrations

The partition coefficients are plotted as abscissae In the early stages of an experiment before an appreciable amount of water has diffused into phase C the rates of entrance of the two salts into that phase are in the same ratio as their partition coefficients Consequently the straight dashed line in the figure indicates the accumulation relative to cesium, in the early stages As the experiment proceeds however, the simple relations which hold initially are complicated by the simultaneous diffusion of water into C This complication has been taken into account in computing the accumulation to be expected in the steady state, as shown by the full curve The circles are the experimental values for systems which have not yet attained the steady state and lie entirely within the area bounded by the full and dashed lines as required by the theory

Thus for the system involving cesium and sodium, the concentration of cesium in phase C in the early stages of the experiment is about 7 8 times that of sodium In the steady state however, the Cs/Na ratio is only 2 7 The observed value of this ratio in an experiment which had not reached the steady state was 4 5, thus being intermediate between the initial and steady state values

V

DISCUSSION

The theory, in its present state of development, has been designed to account for the results of diffusion in the cell models of Osterhout and associates   These models were developed in order to establish a possible mechanism for certain of the effects which are observed in the accumulation of electrolytes by living cells   The behavior of *Valonia macrophysa* is a rather marked example of preferential accumulation of potassium ion from sea water and the cell is able to effect this accumulation without simultaneously developing a total salt concentration in the sap which is very different from that of the surrounding sea water   The following discussion of this cell in terms of the present theory may be of interest, although it cannot be too strongly emphasized that the theory in its present form is quite incapable of accounting for the fact that the chloride ion is the ionic species chiefly associated with the potassium and sodium ions in both the cell sap and the sea water   Disregarding this limitation of the theory, however, and focussing attention upon the concentration relations of the cations, the restrictions which are placed upon the various parameters by certain physical requirements may be investigated as follows

If the subscript 1 refers to potassium and 2 to sodium, the composition of sea water in these two constituents may be taken as $N_1^A = \frac{0\ 012}{55\ 5} = 0\ 000212$, $N_2^A = \frac{0\ 50}{5\ 55} = 0\ 0090$, and $N_1^A/N_2^A = 0\ 024$ The composition of the sap of *Valonia macrophysa*[8] is $N_{I_s} = \frac{0\ 50}{55\ 5} = 0\ 0090$, $N_{II_s} = \frac{0\ 09}{55\ 5} = 0\ 00162$, and $R_s = 5\ 55$ Substitution of these values in equations (3 6) and (3 8) gives the following two relations between K, $P_{1/2}$, and $P_{1/0}$,

$$K = \frac{P_{1/2} - 1}{0\ 024\ P_{1/2} - 5\ 55} \tag{5 1}$$

$$K = \frac{P_{1/0}}{0\ 024\ P_{1/0} - 0\ 00281} \tag{5 2}$$

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[8] Osterhout, W J V, *J Gen Physiol*, 1930–31, **14**, 285

These three parameters are essentially positive and K has been as sumed finite but greater than unity   In equation (5 1), however, K is less than unity for $0 < P_{1/} < 1$ and becomes negative for $1 < P_{1/2} < 231$ After passing through a discontinuity at $P_{1/2} = 231$, the value which renders the denominator of equation (5 1) equal to zero, the value of K then decreases with increasing values of $P_{1/}$ and hence 231 represents a lower limit to the physically possible values for $P_{1/2}$ In a similar manner a lower limit of 0 117 may he assigned to $P_{1/0}$

It will be recalled that $P_{1/2} = \dfrac{D_1 S_1}{D_2 S_2}$, and similarly for $P_{1/0}$ The variations which are observed among the diffusion coefficients of different crystalloids are relatively slight and, considered alone, are probably incapable of accounting for any pronounced deviations of the above ratios from unity   In fact, for the purposes of the present discussion, all diffusion coefficients may be assumed equal, so that $P_{1/} = S_{1/} = S_1/S_2$, and $P_{1/0} = S_1/S_0$ On the other hand it should be possible to find potassium and sodium salts of the same anion whose partition coefficients in a two phase liquid system differ by a factor of several hundred and consequently the minimum value for $S_{1/2}$ which was computed above is physically possible   On the basis of similar arguments, $S_{1/0} > 0\ 117$ is not unreasonable

If the value of 100 be assigned *arbitrarily* to K, values of 396 and 0 2 may be computed for $S_{1/2}$ and $S_{1/0}$, respectively, from equations (5 1) and (5 2)   Since examples are rare in which one of the components in a two component system in two liquid phases has a concentration greater than 0 5 in the phase which is richest in the other component, it is therefore probable that $0 < S_0 < 0\ 5$ If a value of 0 2 be assigned *arbitrarily* to S_0, then $S_1 = 0\ 04$, and $S_2 = 0\ 0001$, values which are physically possible, although indicating a rather low concentration of the potassium and sodium salts in the non aqueous phase   Hence, an application of the incomplete theory to the rather extreme example of preferential potassium accumulation which is presented by *Valonia* does not involve any physical absurdi ties although this fact, of course, does not establish the validity of the mechanism which has been assumed

SUMMARY

The differential equations describing diffusion in cell models have been extended to include the simultaneous penetration of water and two salts   These equations have been solved for the steady state Values for the concentrations in the steady state which may be computed from the equations compare favorably with the experimental values obtained by Osterhout, Kamerling, and Stanley   Moreover, it has been shown elsewhere[5] that the solution for the steady state is essential to a discussion of the volume change or "growth" of phase C in the models and, by analogy, in living cells

THERMAL RECEPTION IN FISHES

By MORTON A. RUBIN

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I

The results of several investigators along with the fact that spinal cutaneous nerves of catfish showed no impulses for temperature changes between 0 and 35°C led Hoagland to suggest that certain special receptors of the lateral line system may possibly serve as thermal receptors

Wells (1914) has found that fish respond quickly to temperature differences in the water and evidently possess thermal receptors   Ward (1927 1929 1932) has shown that temperatures of streams play an important rôle in directing the migrations of salmon   It has been shown by Parker (1902, 1904), Hofer (1908), Parker and Van Heusen (1917) Hoagland (1932, 1933 a, b, c), and Dykgraaf (1933) that the lateral line organs in fishes serve primarily as mechanoreceptors Records of electrical responses from the lateral line nerves of fishes obtained by Hoagland, and described in the papers just cited also show that certain receptors of the lateral line system are normally in a state of continuous activity, discharging impulses repetitively over their attached nerve fibers   These receptors were shown to be supplied by different nerve fibers of the lateral line trunk from those supplying the purely tactile receptors   Relationships were established between the frequency of the spontaneous ' discharge of nerve impulses and the temperature of the receptors (0 to 35°C)   The frequency was found to increase with temperature according to the Arrhenius equation   Thresholds of thermal activation were demonstrated for the discharge of these special organs of the lateral line system in the catfish, *Ameiurus nebulosus* Les

With the view to investigating the rôle of the spontaneously active units as thermal receptors, the following behavior experiments were performed on *Ameiurus nebulosus* Les (the common catfish), *Eupomotis gibbosus* L (the common red gilled sunfish), *Lepomis pallidus* Mitchill (the blue gilled sunfish), and *Perca flavescens* Mitchill (the common perch)   Three groups of each species were used (1) *Normal*—intact fishes, (2) *Cut*—fishes with lateral line nerves (ramus

lateralis X) cut just posterior to the gills on each side of the trunk, (3) *Control*—animals whose skin was cut in the same place as those in Group 2, but with the lateral-line nerves left intact

Modification of behavior in response to thermal stimulation was tested as a function of the lateral-line system in the trunk only, the effect of the head organs on the behavior of the fish was not tested The head organs were left intact in all of the groups, and their effect, if any, upon response to changes in temperature was the same in all the experiments

Animals were placed, one at a time, in an aluminum pan containing water in which was immersed a calibrated thermometer The water was heated by means of an ordinary circular gas burner, and the fish's reactions to the rising temperature were noted Extraneous conditions were kept constant throughout all the experiments The water was changed before each trial The behavior of the fish was independent of the rate of change (approximately 3 to 5°C per minute) of temperature of the bath for the experimental ranges Varying temperature ranges were used between the limits of 0° and 34°C

II

The normal and control fishes became markedly active at specific "response temperatures" indicated in Table I, while the cut fishes became active only at considerably higher, lethal temperatures (37 0 ±1 5°C), showing no activity at the response temperature of the normal fish The responses were in the form of vigorous swimming movements and pushing against the walls of the container At temperatures below the response temperatures the animals were, as a rule, quiescent in the water

On repeating the experiments on a second group of catfish a year later, it was found that they did not respond consistently to the response temperature previously observed The catfish were very restless and would not remain quiet long enough for the experiment to be carried out Freshly caught, young catfish behaved similarly No satisfactory reason could be found for this difference in behavior A second group of red-gilled sunfish, however, behaved typically, and the mummichog (*Fundulus heteroclitus*), showed a characteristic response temperature Instead of being placed directly in the pan,

TABLE I

	Catfish			Sunfish (red gill)			Sunfish (blue-gill)			Perch			Fundulus		
	Response temperature (°C)	No. of trials	No. of fish	Response temperature (°C)	No. of trials	No. of fish	Response temperature (°C)	No. of trials	No. of fish	Response temperature (°C)	No. of trials	No. of fish	Response temperature (°C)	No. of trials	No. of fish
Normal	27 6 ± 0 29	32	4	27 0 ± 0 18	36	4	27 7 ± 0 20	40	4	27 5 ± 0 13	28	3	26 7 ± 0 10	24	3
Cut	None	30	3	None	37	5	None	30	3	None	30	3	None	24	3
Control	27 3 ± 0 20	33	4	26 8 ± 0 18	24	3	27 4 ± 0 05	24	3	27 5 ± 0 02	30	3	26 8 ± 0 05	21	3

Table I shows the mean specific temperatures of response observed, with probable errors based on the number of trials as indicated. The cut fish became violently active at 37 0±1 5°C and died quite soon after being heated to this temperature. The response temperatures of the cut fish are indicated by "none," since in the experiments they were heated only to about 34 0 C in order that more than one trial could be made on each fish

Fundulus was confined in a small beaker which was put in the pan This did away with the random swimming movements which took place when the small *Fundulus* (about 1 5 inches) was placed in the pan The second group of catfish were, however, not quieted by this procedure The results of the experiments on *Fundulus* are also contained in Table I

The behavior of the normal and control sunfish is especially interesting since they usually keel over laterally in response to a slight increase of temperature even before the thermometer records the change The cut sunfish did not keel over in this way, although it was still capable of doing so This indicates that the response is due to the activation of thermal receptors, since the fish could not have been warmed internally to any appreciable extent before a change in temperature was recorded by the thermometer

From this last fact and from the data in Table I it appears that certain receptors of the lateral-line system are involved in thermal reception, probably in addition to those receptors concerned with mechanoreception, since fishes with the rami lateralis X cut do not react to temperature changes as do the intact (normal) or the control fishes

SUMMARY

The reactions of catfish, sunfish, perch, and mummichog to temperature changes, as described in this paper, indicate that certain receptors supplied by the lateral-line nerve are concerned with thermal reception

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STUDIES ON THE GROWTH HORMONE OF PLANTS

VII The Fate of Growth Substance in the Plant and the Nature of the Growth Process

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Since the early work of Went (1928), it has been known that the growth substance of the *Avena* coleoptile may be obtained in the usual way, *i e* by diffusion into agar blocks, only from that part of the plant which produces it, namely the tip   The hormone is not recoverable in this way from those parts of the plant, such as the lower zones, which only make use of it   Recent work (Thimann, 1934) has, how ever, shown that by extraction with chloroform some growth sub stance is recoverable from the lower zones of the coleoptile   The re sults of this extraction method confirmed the earlier view that there is a marked concentration gradient of growth substance from tip to base

The fact that there is less growth substance in the rapidly growing middle zones than in the hormone producing tip has been commonly interpreted to mean that growth substance is actually destroyed in the growth process   It is clear, however, that this is not of necessity the case   It could equally well be that this inactivation is carried out in the plant by processes quite apart from those of growth   In an earlier paper (Thimann and Bonner, 1933), it was shown that the growth of the coleoptile is under certain conditions proportional to the amount of growth substance entering the plant   If it could fur ther be established that, at least under some conditions, the growth appearing is proportional not only to the amount entering but to the amount actually inactivated, this would indicate strongly that the hormone is destroyed, or at least transformed, in the growth process To show that this is actually the case, the extraction method, which

makes it possible to determine the amount of growth substance destroyed, has been used, and experiments of this kind will now be described

EXPERIMENTAL

The chloroform extraction of coleoptiles was used as described in a previous paper, (Thimann, 1934)   The previous procedure was modified by the use of a specially designed mechanical grinder   From the coleoptiles used the tip, 5 to 7 mm long, was in all cases removed   About 200 plants were used for each extraction

In the course of the experiments, considerable variation in the amount of growth substance present in the plants was encountered   This variation is apparently in part normal, but is also in part due to the presence of variable small amounts of peroxides in the chloroform   These quantities were too small to be detected by the titanium sulfate test (less than 5×10^{-5} mols per liter), but were nevertheless sufficient to inactivate small amounts of growth substance, occasionally even a significant fraction of the total amount   That there were small amounts of peroxides present was shown by the partial inactivation of known small quantities of growth substance by shaking with the chloroform   Redistillation, washing with water, and addition of titanous sulfate to the chloroform did not completely remove this power of inactivation

RESULTS

(A) Growth Substance Used and Growth Resulting, after Decapitation —The first point to be investigated was the decrease of growth substance in the plant after the removal of the hormone-producing tip It is known that for approximately 2 hours after removal of this tip the growth rate falls steadily   At the end of this time, the topmost zones of the stump begin to produce the hormone and an increase in the growth rate is observed   Table I shows that correspondingly after 2 hours the amount of growth substance in the plant has fallen to about one-half of its original value   After 3 hours, on the other hand, the amount of growth substance is, due to the "regeneration" described above, approximately the same as in the freshly decapitated plant

Table I shows that in 2 hours approximately 2 7 units of growth substance disappear   The data of the earlier paper (Thimann and Bonner, 1933) show that over the range in which the growth response is proportional to the growth substance entering the plant, 2 7 units

of growth substance in the plant would give 0 73 mm of elongation
If growth is proportional to the amount of growth substance which
disappears, and if all growth substance entering the plant ultimately
disappears in this way, these decapitated coleoptiles should then
grow 0 73 mm   Reference to Table II of the earlier paper reveals
that in 2 hours approximately 0 79 mm of growth occurs, a very satis
factory agreement with the expected value

(B) *Growth Substance Used and Growth Resulting, after Application*
of Additional Growth Substance —If growth substance be applied, in
agar blocks, to a decapitated coleoptile, an immediate increase in
growth rate occurs   In addition the coleoptile continues to grow
faster than normal for some time after removal of the block   In the

TABLE I

Amount of Growth Substance Present in Coleoptiles at Different Times after
Decapitation

Experiment	Time after decapita tion	Growth substance units per plant	Time after decapita tion	Growth substance units per plant
			h s	
1	0	5 0	2	3 1
2	0	6 4	2	2 9
3 (Mean of two)	0	7 7	3	6 4

present experiments, agar blocks containing 47 units of growth sub
stance were allowed to remain upon coleoptiles during 2 hours   Dur
ing this time approximately 37 per cent (extrapolation from data for
110 minutes in Table I of Thimann and Bonner, 1933), or 17 4 units
of this 47 units, enter the plant   Table II shows that, however, only
a small proportion of this may be recovered at the end of 2 hours   The
average of several experiments (see also Table III) gives 6 6 units as
the amount recoverable   According to the earlier paper, this 6 6 units
should cause a further 1 78 mm of elongation before the coleoptile
returns to the normal growth rate   Interpolating in Table III of
the same paper, (1933), (for 47 unit blocks), one finds that approxi
mately 1 72 mm occur, again a very satisfactory agreement with the
expected value

We have now two cases in which the growth appearing is proportional, not only to the growth substance entering the plant, but also to the growth substance used up   In these two cases it appears, then, that the growth is proportional to the amount of growth substance which has undergone transformation into an inactive form

In the earlier paper it was shown that with agar blocks containing as much as 47 units, the growth per unit of growth substance is less than

TABLE II

Amount of Growth Substance (in Units per Plant) Recoverable from Coleoptiles Supplied with 47 Unit Agar Blocks during 2 Hours

Experiment	Plants with 47 unit blocks	Plants without 47 unit blocks
1	9 4	3 7
2	3 5	1 7
3	5 5	2 4

TABLE III

Average Number of Growth Substance Units Recovered from Freshly Decapitated Plants and from Plants Supplied during 2 Hours with 47 Unit Agar Blocks

Units per plant	
Fresh plants	47 unit plants
5 0	9 4
6 4	3 5
4 2	5 5
3 2	8 7
3 4	5 0
3 3	7 6
Mean 4 3 ± 0 5	6 6 ± 0 9

with smaller amounts in the block, i e , some factor other than growth substance is limiting growth   Considering the first 2 hours of the present experiment, 17 4 units will enter the plant from the block   In addition there were originally present 4 3 units, (Table III)   During the 2 hours, then, 21 7 less 6 6, or 15 1, units of growth substance have disappeared   Although if the response to growth substance were linear this would result in 4 06 mm of growth, Table III of the earlier

paper (Thimann and Bonner, 1933) shows that only 2 55 mm appear
There is, then, in the presence of high growth substance concentrations,
more growth substance destroyed than is used in growth   This was
confirmed by the application for 2 hours of blocks containing 310
units   At the end of the 2 hours, the plants were found to contain only
9 6 units per plant   The amount of growth in plants with 310 unit
blocks is only about 25 per cent more than in plants with 47 unit
blocks, while the amount of growth substance inactivation is about 7
times as much   Thus, in this case, the bulk of the growth substance
entering the plant is destroyed without resulting in growth   This
excessive destruction of growth substance in the presence of high
concentrations is more than sufficient to account for the deviation from

TABLE IV

Extraction of Growth Substance from Cut Off Coleoptiles

| Treatment | Plants with bases in water | | Plants with bases dry | |
| | Growth substance in units per plant | | Growth substance in units per plant | |
	Before treatment	After treatment	Before treatment	After treatment
Decapitate leave 2 hours	3 2	1 6	4 2	1 9
Apply 47 unit block for 2 hours		8 7		7 0
		5 0		6 0
Mean		6 8		6 5

linearity of the growth growth substance curve given in the earlier
paper

(C) *Growth Substance Used without any Accompanying Growth* —
From the preceding section it is necessary to conclude that in the pres
ence of small growth substance concentrations and of external con
ditions favorable to growth there is a quantitative relation between
growth substance converted to an inactive form, *i e*, used up, and
growth resulting   In the presence of excess growth substance, how
ever, the latter disappears without the appearance of a corresponding
amount of growth   This fact suggests that if factors other than
growth substance were made to limit growth the hormone would never
theless disappear   Experiments were therefore carried out in which

available water was made the limiting factor   In Table IV is summarized the disappearance of growth substance in coleoptiles cut from their roots and placed (1) with their bases in water, and (2) with their bases dry, in a saturated atmosphere   The decrease of growth substance after decapitation takes place to about the same extent as in the normal plant in both cases   In addition, if 47 unit blocks be applied to these plants for 2 hours, then the amount of growth substance which disappears is about the same as in normal plants, despite the fact that, as will be shown in Table V, the plants with inadequate water supply grow only about one-fourth as much as those with their bases in water   The logical conclusion is that under conditions unfavorable to growth the conversion of growth substance to an inactive form nevertheless takes place

(D) *Growth Substance Used in Relation to Subsequent Growth* — It is of interest to discover whether this growth substance which disappears without any accompanying growth is available for subsequent growth if the conditions are again made favorable   For this purpose the growth, in 2 hours, of plants to which 47 unit agar blocks were applied, with and without adequate water supply, was measured   At the end of this time water was supplied to all the plants and the agar blocks removed from one-half of the plants of each group   The growth rates were then measured during the next 4 hours   We know that in the "wet" plants there was in the first 2 hours a certain amount of growth substance converted to inactive form and presumably used, at least in part, for growth   In the "dry" plants, on the other hand, the same amount of growth substance has been destroyed in the first 2 hours but a much smaller portion of it used in growth since less growth has occurred, (Table V)   If the uselessly destroyed growth substance is still available for growth, these formerly dry plants should, upon the addition of water, grow faster than the previously wet plants   Table V shows that on the contrary they grow more slowly   This is not because they are for other reasons incapable of fast growth, since those to which growth substance is continuously supplied grow much more swiftly   It is necessary to conclude that growth substance, in order to be used in growth, must be inactivated at the time at which the actual growth takes place

(E) *Nature of the Inactivation Reaction* —Growth substance is

known to be readily oxidized *in vitro* both by ordinary oxidants (e g hydrogen peroxide) and by plant oxidase preparations, (Thimann, 1934)   It is also known that at least one of the growth processes

TABLE V

Effect of Temporary Water Lack upon the Growth of Coleoptiles (Agar Blocks Containing 47 Units)

Bases wet		Bases wet		No. of plants
Growth in 1st 2 hrs.		Growth in		
		2nd 2 hrs.	3rd 2 hours	
per cent		*per cent*	*per cent*	
6 6	Blocks left on	8 4	5 5	9
	Blocks taken off	4 0	0 3	9

Bases dry		Bases wet		No of plants
Growth in 1st 2 hrs.		Growth in		
		2nd 2 hrs.	3rd 2 hrs.	
per cent		*per cent*	*per cent*	
1 6	Blocks left on	5 8	4 1	7
	Blocks taken off	2 0	0 8	9

TABLE VI

Disappearance of Growth Substance in the Presence of 10^{-2} N HCN

| Experiment | Growth substance (units per plant) in plants | | |
	Freshly decapitated	In HCN 2 hrs.	In water 2 hrs
1	2 1	0 4	0 3
2	3 0	3 0	2 2
3	4 8	0 8	0 8
4	3 7	1 6	1 8
Means	3 4	1 4	1 3

of the coleoptile can be inhibited by HCN in the same manner as can the respiration (Bonner, 1933)   It was therefore of interest to determine whether the inactivation of growth substance in the plant is inhibited by HCN   Table VI shows that the destruction of growth

substance is not significantly affected by concentrations of HCN sufficient to inhibit growth almost completely The destruction of growth substance is not, therefore, the cyanide-inhibited reaction of the growth process

DISCUSSION

The reactions taking place in the cell elongation of the *Avena* coleoptile may be included under the following heads

1 The Passage of Growth Substance into the Growing Portions of the Plant —This process has been studied particularly by Van der Wey (1932, 1934) It has been shown not to be a simple diffusion of the hormone Unpublished experiments have shown that in the absence of oxygen the transport of growth substance does not take place It is clear that the transport of the hormone from the tip or agar block is a prerequisite for the reaction of growth substance in the growing regions of the plant

2 The Chemical Transformation of Growth Substance —It has been shown above that under conditions which are favorable to growth, growth substance inactivation is strictly proportional to growth It is therefore most reasonable to assume that the chemical transformation involved is an essential reaction in the growth process Under conditions unfavorable for growth the inactivation of growth substance nevertheless continues This is most easily interpreted to mean that the inactivation is the first member of a chain of growth reactions, and that hence it may take place even if the subsequent reactions do not Concerning the nature of the change we have no information other than that it is not inhibited by cyanide It has been shown above that, in order to be of use in growth, this chemical transformation must take place at the same time as the actual elongation, *i e*, the products of the reaction cannot be stored for any appreciable time This reaction is, then, closely linked to those succeeding it

3 The Cyanide-Inhibited Reaction —It has been shown (Bonner, 1933), that the growth of the *Avena* coleoptile is inhibited by HCN in approximately the same way as is its respiration Since, however, most of the energy of respiration is liberated as heat, the respiration as a whole can hardly be said to be a part of the growth process On the contrary it may be said with fair certainty, on this as well as on

other grounds, that the respiration like reaction of the growth process forms but a small portion of the total respiration   It has been shown above that the inactivation of growth substance is not the reaction which is inhibited by HCN, but that these two distinct reactions are necessary to the growth process

4 The Physical Process —This portion of the growth process is probably the actual mechanical stretching of the cell walls   Some experiments on temperature coefficients, which will not be given in detail here, indicate that the Q_{10} of coleoptile elongation as a whole is markedly below 2, at least for some time after removal of a coleoptile from a higher temperature to a lower   During the first 2 hours after decreasing the temperature, the average Q_{10} between, for example, 25° and 10° was found to be 1 7 while the Q_{10} of the respiratory reaction, as measured by the Q_{10} of the respiration as a whole, was under the same conditions 2 6   This indicates that the respiratory process with its high Q_{10} is linked to a process having a very low Q_{10}, perhaps close to 1, *i e* , a physical process   It is of interest that Heyn and Van Overbeek (1931) have shown that the Q_{10} of plastic stretching of the cell walls of the coleoptile is also below 2

5 The Uptake of Water and the Resulting Visible Increase in Cell Size —It is of course clear that water uptake is necessary for an increase in cell size   This is well illustrated in Table V, where plants having an inadequate water supply elongate but little

If reaction 4 is the mechanical stretching of the cell wall, it is clear that 4 and 5 must go on at the same time   In Section *D* above it was shown that reaction 2 must go on at the same time as 4 and 5, and it has been previously and independently shown that reaction 3 must take place at the same time as reactions 4 and 5   The last four components of the growth process are, therefore, closely linked

Heyn (1931) and Heyn and Van Overbeek (1931) have shown that the principal change in the properties of the coleoptile which accompanies the action of growth substance is an increase in the plasticity of the cell wall   Since reactions 4 and 5 are concerned with the actual elongation, it is clear that this increase in wall plasticity must take place as a *result* of reactions 2 and 3

In view of the fact that, up to the present, only information of a rather negative nature has been obtained concerning the more inti

mate qualities of the above components of the growth process in the *Avena* coleoptile, a further discussion would be unprofitable It is of interest, however, to summarize in a diagram the reactions which are already known, particularly for comparison with the schemes recently proposed by Soding (1934) and by Strugger (1934) It is clear from this diagram that the growth process may be stopped at any one of a number of places When it is so stopped, the reactions preceding the one which is inhibited may continue to take place, but no growth occurs

The Growth Process

	Preliminary	Preparation of the cell wall		Visible growth	
The reactions	1 ———→ 2 Growth substance transport and uptake by the cell	Growth substance transformation	———→ 3 Reaction inhibited by HCN	———→ 4 ←——— Physical or mechanical process (wall stretching)	5 Water uptake (or other mechanical force)
Growth is inhibited by	1 Lack of growth substance Lack of oxygen		3 Presence of HCN Lack of oxygen		5 Lack of water

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ON THE GEOTROPIC ORIENTATION OF HELIX

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I

Concerning the control of geotropic orientation in pulmonates there has existed a certain difference of opinion With *Helix aspersa* Cole (1928) showed that the speed of oriented creeping upon an inclined surface was greater the higher the slope of the surface, and that this speed was increased by forcing the snail to carry an added load   Wolf (1926–27) found that *Agriolimax* oriented upward upon an inclined surface in such a way that the angle of the path upon the surface increased with the slope, in the general manner found for geonegative orientation in young rats (Crozier and Pincus, 1926–27, etc) and a variety of other forms (*cf* Crozier, 1929)   On the basis of certain perhaps unnecessary and in all probability irrelevant preconceptions as to the significance of the statocyst, and of certain tests which will be discussed subsequently, Piéron (1928), von Buddenbrock (1931), and Jäger (1932) have objected to the interpretation of these and related facts, particularly as concerns the geonegative orientation of *Limax* and *Helix*

A variety of tests with diverse pulmonates demonstrates the preponderant rôle of the distribution of tensions impressed asymmetrically upon the body musculature in the primary excitation leading to geotropic orientation (Cole, 1928, Crozier and Federighi, 1924–25, Wolf, 1926–27, Crozier and Pincus, 1929–30, Crozier and Navez, 1930, Hoagland and Crozier, 1931–32)   The further fact that, as demonstrated with a number of forms, there is a relationship between the slope of the surface and the angle of oriented progression upon it (Crozier and Navez, 1930) implies that the process of orientation continues until there is sensorial equivalence of the effects of muscle

stretching upon the two sides of the body   On this assumption equations have been arrived at (*cf* Crozier and Pincus, 1926–27, etc , Wolf, 1926–27) which successfully describe the connection between angular inclination of surface (α) and angle of orientation on the surface (θ)   The curves depicting these relationships are distorted in predictable ways when the animals are forced to carry additional loads, and in other ways, and the equations are necessarily different for animals which are geometrically diverse as regards the distribution of gravitationally impressed tensions in the supporting musculature (*cf* Crozier and Stier, 1927–28, 1928–29, Kropp and Crozier, 1928–29, Crozier and Pincus, 1929–30)   (It must be remarked that to these facts, and to the functional analysis of the *variability* of geotropic performance in the papers cited, Piéron (1928), von Buddenbrock (1931), and Jager (1932) give no attention whatever)

Upon an inclined surface the gravitational component capable of influencing the orientation of a pulmonate is proportional to sin α It happens that for *Agriolimax* and certain other animals the angle of upwardly directed creeping is nearly (although not exactly) proportional to log sin α (Wolf, 1926–27, Crozier and Pincus, 1926–27, 1929–30), the curve is in fact a flat *S*, although closely spaced observations are required to detect it   From reasonable assumptions as to the distribution of the pull of the animal's mass upon the anterior musculature, the equation

$$(\Delta \sin \alpha)(\Delta \sin \theta) = - const$$

was derived (*cf* Wolf, 1926–27) to describe the limitation of orientation in *Agriolimax* as a function of the slope of the surface, and does so successfully

It was desirable to reexamine this matter, particularly with *Helix* Jager (1932) concluded that since *Helix* has a statocyst, and since it appeared to orient precisely under water, with no dependence of θ upon α, the law of limitation of orientation through equilibration of muscle tensions on the two sides could not apply   Hoagland and Crozier (1931–32) had found, however, that the reaction time (latent period) for geotropic response was inversely proportional to sin α, and was shortened by added loads (as in *Liguus* (Crozier and Navez, 1930))

II

Helix nemoralis were prepared a day before by removing the eye bearing ends of the cephalic tentacles With the foot extended for creeping a snail was then transferred to a large freshly washed glass plate inclined at a desired angle The long axis of the snail was placed in a horizontal line When creeping was begun orientation upward occurred at all slopes above 15 The *oriented* path was at an angle θ on the glass plate, measured by placing on the under side of the plate a celluloid ruler with a central line marked upon it which permitted the rapid use of a protractor There were frequently slight differences apparent between orientations to the right and to the left For convenience, only orientations to the right were considered The path is easily disturbed by air currents due to motion of the observer's hands, or to the breath In creeping there occur alter nating slow turnings of the anterior end to right and to left, which bring about a slight time fluctuation of orientation angle in repeated trials at brief intervals If initially so placed that orientation involves turning to the left, the snail frequently swings ' too far,' and the final path is oriented to the left—that is, as if it had been started with the right side up If at the start of creeping the head is lifted and moved about, the first part of the upward trail is likely to be too steep as the anterior end of the foot becomes attached and initiates creeping at the point on the inclined glass which is encountered by the head, if allowed to creep far enough however the orientation angle falls to that characteristic for the particu lar slope After repeated handlings downward orientation is apparent at $\alpha = 15°$ or even at $\alpha = 20°$, increasing the tilt of the surface however always results in prompt upward orientation When this reversal' to downward creeping is apparent the body is much extended, as in tests under water The reversal is perhaps related to that seen in *Liguus* with too heavy loads (Crozier and Navez, 1930) Orientation angles were also measured by tilting a plate initially hori zontal upon which *Helix* was creeping freely this more tedious method gave the same θ s as the other At a given slope θ is slightly higher when the snail creeps faster

Similar tests were made at three slopes with the snail adhering to the *under* surface of the glass plate (Table I) As with *Agriolimax*, θ is the same, at the same slope, as when the snail is on the upper surface (cf Wolf, 1926–27)

If by means of a thread the shell is held on the upper side of the snail while orientation is being started the head does not turn upward and creeping con tinues horizontally Occasionally the shell is held in this position spontaneously and horizontal creeping is evidenced even at $\alpha = 40°$ if the plate be then rotated so as to bring the other side uppermost, orientation is prompt Such facts speak unmistakably for the determination and control of orientation by the pull of the shell and visceral mass If this pull be increased by attachment of a bit of lead weighing about 1 gm to the front of the shell, θ at $\alpha = 20°$ is increased from *ca* 45° to 64 6° ±1 82 (It is of interest that for this case also $P E_{\theta}$ plotted against θ shows in Fig 4 that the variation of θ is a definite function of the intensity of excitation)

These facts are unequivocally consistent with the view that the determination of θ as a function of α is not due to any operation of the statocyst   Jager (1932), however, states that when *Helix* creeps under water (reducing pull of the shell), θ is approximately 90° at all slopes of surface, as Piéron had claimed (1928) for *Limax* We have earlier pointed out (Crozier and Navez, 1930, Crozier and Pincus, 1929–30) that with *Liguus* and *Lunatia* θ is less at given α under water, and that with *Helix* and *Limax* it is difficult to make really significant tests   Particularly if the posterior portion of the foot is not adherent, the directly upward pull of the floating shell makes it necessary for θ to be greater if bilateral equalization of tensions is to be brought about, the total downward pull is reduced, but simple consideration of the geometry of the situation shows that until orientation is achieved the ratio of tensions on the two sides is augmented   The situation is the same in young rats to which a gas-filled balloon has been attached at the mid-point of the back, and here also θ is *increased*   When *Helix* is under water, moreover, the body is much extended, and mechanical irritability seems much increased   Nevertheless, tests in which *Helix* attached to a horizontal plate are lowered into the water and the plate subsequently tilted do show a *lowering* of θ, provided the foot continues to adhere and if creeping continues   The test succeeds better if the shell be weighted with lead, so that it is not too buoyant   (If the upward pull of the too buoyant shell is too strong, there may be a "reversal of geotropism," as shown in *Liguus* (Crozier and Navez, 1930), which would, however, lead to upward creeping)   A well attached *Helix*, under water, is easily directed by gentle traction upon the shell to one side or the other   For these reasons we conclude that the argument advanced by Piéron and by Jager is without real foundation

<div align="center">III</div>

Mean orientation angles (θ), and the indices of dispersion of θ, are given in Table I   Fig 1 shows that with sufficient exactness θ is a nearly rectilinear function of log sin α, as with *Agriolimax* (Wolf, 1926–27)   von Buddenbrock (1931) has suggested that the orientation observed in these experiments is better measured by the angle of the animal's transverse axis in space, i c , by the angle δ, where

$$\sin \delta = \sin \alpha \cos \theta$$

He states that δ "varies very little," Jager (1932) is of this opinion also   This was held to signify that upon an inclined surface the animal "tries to keep its transverse axis as nearly level as possible," rather than that δ is controlled by sensorial equivalence of tensions In fact, however, although δ is necessarily a fairly small angle, it varies much more than does θ—by a factor of 10 when θ changes by a factor

of 3, in some cases, and since θ increases with α it is obvious that δ, far from tending to constancy, must pass through a maximum—as indeed it does in each such series of measurements There is conse quently no basis whatever in the data for the idea that geotropic orientation is limited by selection of a minimum of transverse inclina tion, and it does not seem to have been noticed that the experiments with added loads are completely inconsistent with any such notion

TABLE I

Angle of orientation (θ) as a function of inclination of surface (α) for *Helix nemoralis* negatively geotropic progression Three series of observations, in *1*, the figures in brackets refer to orientation upon the *under* surface of a glass plate Orientation angles are each the average of 12 to 22 observations $P E_\theta$ is corrected

α	θ degrees		
	1	*2*	*3*
degrees			
15	$37\ 1 \pm 2\ 10$ $[32\ 5 \pm 2\ 01]$	$33\ 5 \pm 2\ 77$	
20		$47\ 4 \pm 2\ 97$	$42\ 5 \pm 2\ 60$
23	$58\ 3 \pm 2\ 50$ $[57\ 8 \pm 3\ 11]$		
25	$60\ 6 \pm 1\ 94$		$48\ 3 \pm 2\ 73$
30	$54\ 0 \pm 3\ 41$	$55\ 4 \pm 1\ 67$	
35			$58\ 0 \pm 2\ 19$
40		$67\ 1 \pm 1\ 40$	
45			$66\ 8 \pm 2\ 52$
50	$78\ 2 \pm 0\ 51$ $[72\ 0 \pm 1\ 03]$		
60		$79\ 0 \pm 1\ 44$	
70	$79\ 6 \pm 1\ 32$		
80		$87\ 0 \pm 0\ 26$	
90			$85\ 4 \pm 0\ 81$

The rectilinear relationship between sin θ and $1/\sin \alpha$ was derived for the orientation of *Agriolimax* and similar forms in which the ten sions supposed to be brought into equivalence in a sensory way by the completion of orientation are exerted upon mutually inclined longitudinal elements at the anterior end of the body It is suf ficiently obeyed in the measurements with *Helix* (Fig 2)

When the force responsible for orientation is small, the precision

of orientation must be expected to be less   The chance effects of other sources of stimulation, with their possibilities for central nervous inhibition of the gravitationally induced excitation, are then more likely to affect θ, and the spontaneously occurring changes in geotropic reactivity are likely to have greater effect   There should then appear a relationship of some sort between α and *variation* of θ For the pri-
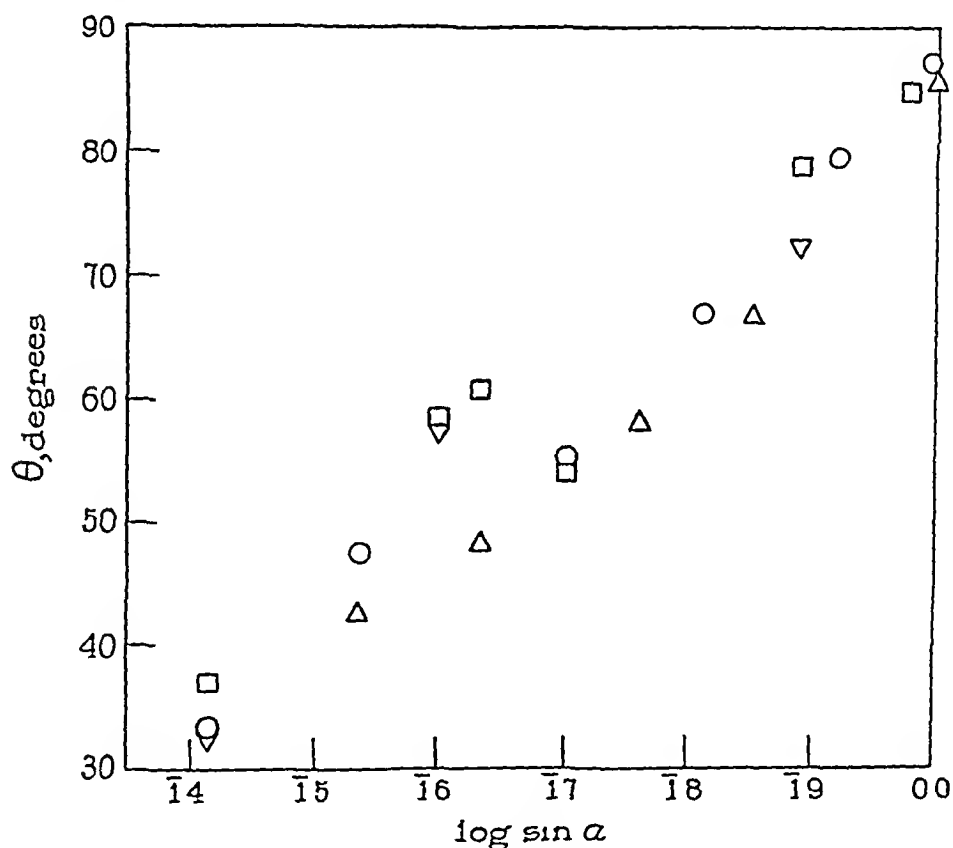
Fig 1 Mean angles of orientation (θ) during upward geotropic creeping of *Helix nemoralis* (cf Table I), as related to log sin α

mary geotropic excitation of *Helix* Hoagland and Crozier (1931–32) demonstrated that $P E_{RT}$ is a straight-line function of $R T$, where $R T$ is the latent period (reaction time)   For other cases it has been shown (Crozier and Pincus, 1931–32) that σ_* declines rectilinearly as sin α increases, the rule holds in the present instance also (Fig 3) On the other hand the *relative* variation of θ is expected to be a de-
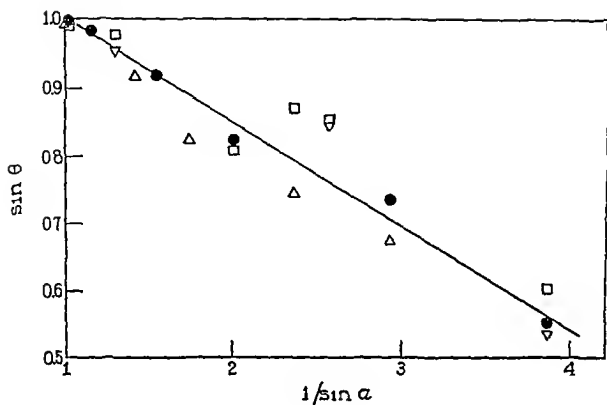
Fig 2 Sin θ is a rectilinear function of $1/\sin \alpha$
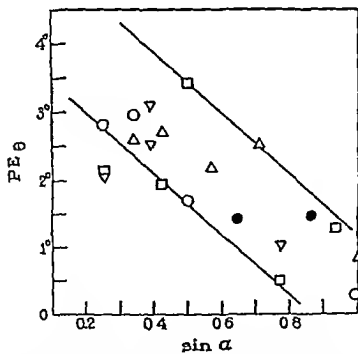
Fig 3 $P E_\theta$ declines regularly as $\sin \alpha$ is increased

clining rectilinear function of θ (Crozier and Pincus, 1926–27, etc), Fig 4 shows that this is found (each of the plotted values is subject to a $P\,E$ proportional to its own magnitude, so that the scatter is greater at low values of θ)
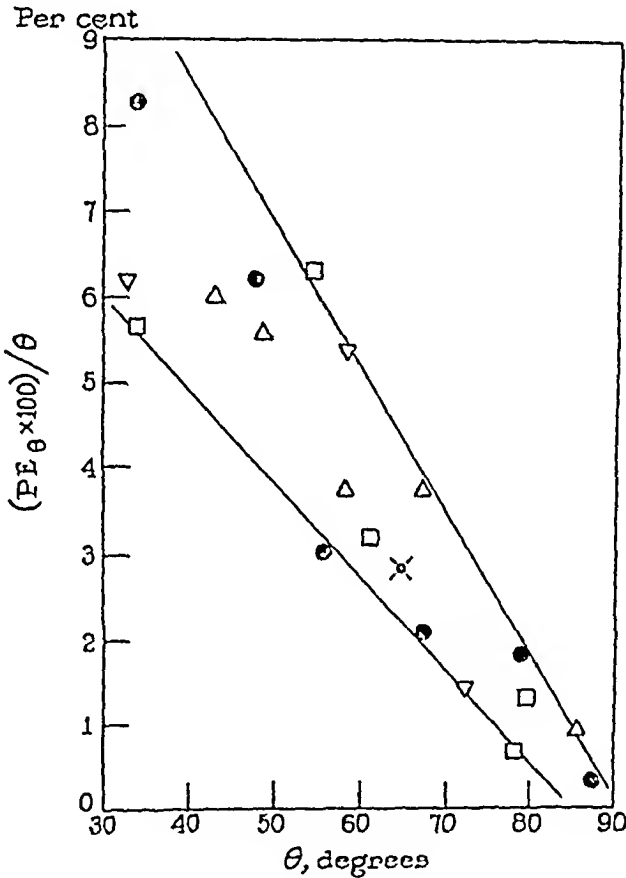
Fig 4   100 $P\,E_\theta/\theta$ declines regularly as θ increases, see text, the $\times$ refers to an experiment with added weight (p 661)

<center>SUMMARY</center>

The snail *Helix nemoralis* in negatively geotropic creeping orients upward upon an inclined surface until the angle of the path of progression (θ) is related to the tilt of the surface (α) as (Δ sin θ) (Δ sin α) $= - const$, θ is very nearly a rectilinear function of log sin α The precision of orientation ($P\,E_\theta$) declines in proportion to increasing sin α, $P\,E_\theta/\theta$ in proportion to θ These facts are comprehensible

only in terms of the view that the limitation of orientation is controlled by the sensorial equivalence of impressed tensions in the anterior musculature

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THE EFFECTS OF RADIATIONS ON BIOLOGICAL SYSTEMS

II Immediate and Subsequent Effects of X Ray Irradiation Upon Respiration of Drosophila Larvae

By RAYMOND HUSSEY and WILLIAM R. THOMPSON

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In gathering data concerning the effect of radiations on biological systems we have proceeded in our study from a simple system, enzymes in solution,[1] to a more complex system, *Drosophila* larvae[4] As a result of irradiation with x ray radiation the prepupal period of the life cycle of the *Drosophila* is prolonged This effect serves as a convenient measure of relative effectiveness of radiations[4] In the hope that *Drosophila* larvae might prove to be a convenient system to study more satisfactorily the immediate as well as more remote effects of irradiation previously attempted in the rabbit[3] and dog, we determined to observe the influence of x rays on metabolic activity as measured by respiratory rates Since no satisfactory method was available for such observations, it was necessary to develop one, which end was attained by the construction of a respirometer capable of use under all conditions of constant humidity The apparatus and the principles

[1] Hussey R., and Thompson, W R , *J Gen Physiol* , 1922–23, 5, 647 1923–24 6, 1, 7 1925–26, 9, 211, 217, 309, 315 Thompson W R , and Hussey, R , *J Gen Physiol* , 1931–32, 15, 9 Thompson, W R., and Tennant, R., *Proc Soc Exp Biol and Med* , 1932, 29, 510

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[3] Tennant, R , *Science*, 1931, 73, 567
[4] Hussey R Thompson W R., Tennant, R , and Campbell, N D *J Gen Physiol* 1932–33, 16, 207
[5] Hussey R , *J Gen Physiol* , 1921–22, 4, 511 Sprunt, D H *J Biol Chem* , 1931, 92, 605

of refractovolumetric respirometry, applied especially to the measurement of CO_2 and O_2 changes, are described elsewhere [6-8]

Technique

Larvae for experiments were obtained in the same manner as in the work recently reported[4] except that seedings were made upon a preparation called Y B A, No 3 instead of yeast-agar as before, and larvae were transferred in the usual manner[4] at a median age, a, of 2 5 days in random samples of approximately equal numbers to ordinary tapering drinking glasses, each of which contained 25 ml of a special food, Y B A, No 2, Y B A, No N (Yeast-banana-agar No N) being a mixture in the proportion 1 gm of over-ripe banana pulp, +3 gm Fleischmann's yeast, +5 ml of No N agar mixture in the proportion 9 N gm agar-agar, +1 gm sodium acetate, + enough water and acetic acid to make the volume equal 1 liter and the pH = 6 0

In the present work aseptic technique was not maintained beyond this point

These vessels were convertible into respiratory chambers suitable for sealing into the respiratory machine by fitting into the tops a large rubber stopper with inlet and outlet delivery tubes, the stopper being fitted into the tumbler about 1 cm below the rim, the space above the stopper partly filled with mercury during observation in order to prevent leakage at the joints or through the rubber   The ends of the delivery tubes projecting into the tumbler were provided with a flange which was covered with a double-layered voile cap in order to prevent the escape of larvae   The method of estimation of CO_2 respiration was essentially that used for *Drosophila* imagos in a water-saturated environment [8]   However, no attempt was made to estimate O_2 changes in these experiments   This was made necessary by our interest in observing respiratory rates in simultaneously maintained groups of larvae from the same lot but variously treated as to irradiation   Respiration in 20 minute intervals was measured, preceded by an introduction of fresh air (approximately water-saturated) and a 10 minute mixing and adjusting period in the manner previously described [6,8]   A set of blanks was used to introduce a correction for failure to attain complete saturation with water in the 10 minutes of preliminary circulation   This correction and its standard deviation played a minor role in the estimation of CO_2 respiration   Indeed, the crude refractivity change observations, if plotted in place of the CO_2 change estimates, give practically the same general impression

EXPERIMENTAL

Prior to irradiation respiratory observations were made over an interval of approximately 1 day, the age at the beginning of irradiation approximating 3 5

[6] Thompson, W R, *J Gen Physiol*, 1932–33, **16**, 5

[7] Thompson, W R, and Tennant, R, *J Gen Physiol*, 1932–33, **16**, 23

[8] Thompson, W R, and Tennant, R., *J Gen Physiol*, 1932–33, **16**, 27

days In irradiations the tumbler, food and larvae were merely covered with perforated wrapping paper The source of radiations was a Coolidge water cooled x ray tube,[4] with target directly above the centroid of three tumblers of the type just described, placed upright with rims contiguous It was impressed with a potential difference of peak approximately equal to 188 kv with a tube current of 30 ma The distance from target center to objective was approximately 59 cm with no filter except a piece of white writing paper placed across the aperture The aperture and all other conditions were the same as in experiments recently reported [4] Irradiations were made to start simultaneously in any given
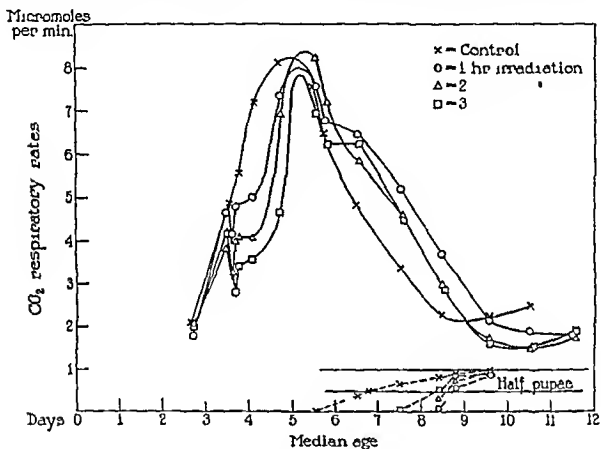
Fig 1

experiment, the various lots being removed at the end of prescribed periods and replaced by dummy tumblers with covers of the same type. The results of two experiments where larvae were irradiated for 1, 2, and 3 hours in this manner (between 22 to 25 C) and CO respiration rates contrasted with those of a control are given in Figs 1 and 2 The larvae chambers were flushed at all times except during transfer or irradiation with a stream of water saturated air at 24 2 C which was fresh except when sealed in the respirometer

An experiment similar to these in all respects (including a control) except that the x ray tube was not operated yielded no significant differences between any of the lots of larvae Thus it is indicated

that the definite differences exhibited in the former experiments were the result of irradiation

Pupation was followed in each instance and the median time of pupation, P, estimated by means of independent inspection estimates of three observers. The coincidental estimates of the fraction, f, of individuals having pupated is given below the respiration curves, using the same abscissae. Thus P is the time where $f = 0.5$ for a given lot
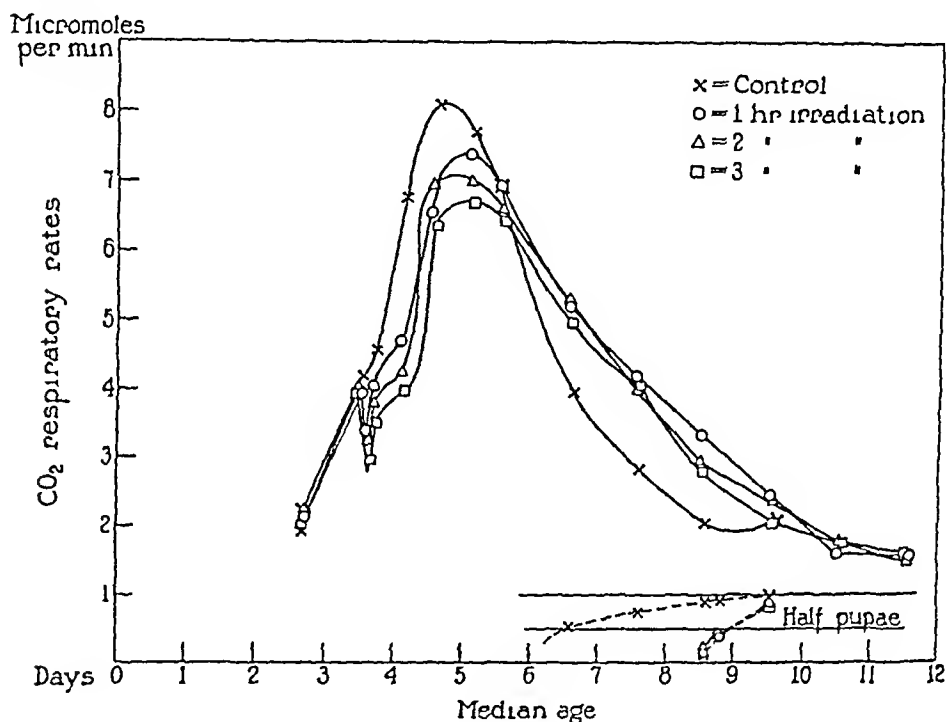
Fig 2

The characteristic most obvious in these experiments is the decreased respiratory rates observed immediately after irradiation. It appears as if the CO_2 respiratory rate of such larvae is a decreasing function of the period of irradiation within the limits of the experiments, but upon discontinuation of irradiation this rate increases rapidly for a short time (about 0.1 day), then markedly less rapidly (with a point of inflexion), then more rapidly again up to a maximum. Apparently the control attains a maximum sooner and declines to a

minimum about a day or two before the irradiated lots—this latent decline being correlated, with the observed delay in pupation   The subsequent rise apparent in the control curves is associated with the emergence of imagos—observation being discontinued on account of their extensive drowning in the remnants of the food   The characteristic decreased rate of respiration after irradiation was observed when larvae were irradiated under essentially the same conditions of maintenance as in former work,[4] that is, in sealed wells in stearin paraffin with perforated tops   Under such conditions, however, it was extremely difficult to measure respiration soon after or before irradiation and considerable manipulation of the larvae at the time seems objectionable and might even confuse the issues involved, hence the resort to the procedure described above   Results similar in some respects but markedly different in others have been obtained in similar manner using ultraviolet light instead of x rays, which will be reported in another communication

COMMENT

It should be emphasized that in these observations our interest in following changes in functional state at short intervals and in simultaneously maintained lots was of prime importance   From this point of view observations of respiration were made without a prolonged interval in which to allow adjustment of the larvae to their altered environment.   In this manner slightly higher respiratory rates pertained than otherwise, but these were consistent provided at least a half hour interval were allowed to elapse between any two observations on the same lot   Therefore, we should not strictly interpret the curves obtained as indicative of actual respiration during the intervals between observations, but we assume that they indicate this relatively

It is of interest, however, to consider relationships between the apparent total CO_2 respiration of the different lots over the interval from the age of 2 5 days to the end of the larval stage   If the result of the action of the radiations upon the larvae were merely to uniformly alter the rates of all processes, then it would follow that the total CO_2 respired would be the same in any interval between corresponding structural states of the larvae, which obviously is not the case

SUMMARY

By means of measurement of CO_2 respiratory rates it has been possible to observe *immediate* and *latent* effects of x-ray irradiations upon *Drosophila* larvae   The observations were extended over a period of several days, the duration of the prepupal period being observed also In every instance a significant decrease in the rate of CO_2 respiration was observed immediately after irradiation   This decrease was univariant with the period of irradiation within the experimental limits

PROTECTIVE ACTION IN IRRADIATION OF AMYLASE SOLUTIONS WITH ULTRAVIOLET LIGHT

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In another communication[1] the monomolecular character of the inactivation of amylase (pancreatin solution) with ultraviolet light has been demonstrated, $i\,e$,

$$\frac{dQ}{dt} = -k\,Q \quad \text{or} \quad \frac{d\log Q}{dt} = -k \tag{1}$$

where Q is the active enzyme concentration,[2-4] t is time in minutes, and k is a constant for given irradiation conditions It was found,[5] however, that the addition of small amounts of dog serum to pancreatin solutions gave marked protection (a speed coefficient, k, of about 1/2 the value for simple pancreatin solutions being obtained by addition of 0 2 per cent of serum) The nature of this protection, whether the result of chemical combination with enzyme in a more stable form or of simple screening of the part of the solution more remote from the source of radiations is of particular interest Certain relations of chemical combination with the enzyme, trypsin, have been studied by Hussey and Northrop,[6] but whether similar results can be obtained in the case of amylase has not been established In further investigation we have obtained evidence of a protective

[1] Thompson, W R. and Hussey R. *J Gen Physiol*, 1931-32 15, 9

Thompson, W R., Johnson C E, and Hussey R *J Gen Physiol* 1931-32 15, 1

[2] Wies, C H and McGarvey S M *J Gen Physiol* 1932-33 16, 221

[4] Thompson W R. McGarvey, S M, and Wies, C H, *J Gen Physiol*, 1932-33, 16, 229

[5] Thompson, W R, and Tennant R., *Proc Soc Exp Biol and Med* 1932, 29, 510

[6] Hussey R and Northrop J H, *J Gen Physiol* 1922-23 5, 335

effect in pancreatin solutions themselves, which is similar to that resulting from addition of serum   A simple competitive absorption theory seems to account satisfactorily for this effect, at least as a first approximation

The method of irradiation was essentially the same as employed in former work[1] [5] except that a different lamp was used   As before,[1] solutions were stirred during irradiations in a cylindrical quartz tube at 10°C   Radiations entering the tube were first filtered through 5 mm of water at 10°C as has been described previously[1]   The source was a mercury arc in quartz, 200 mm below a quartz window in the bottom of the bath   Except in two instances the potential difference across the lamp was maintained at 75 volts, and the current was approximately 4 amperes   In this way several (5) irradiations with a 0 02 per cent pancreatin solution as used in the former work,[1] [5] except that a standard content[7] of 3/10 of the amounts of phosphate buffers required for pH $= 6\ 7$ was adopted, gave a mean speed coefficient, $\bar{k} = 0\ 259$ with a d $= 0\ 004$ In contrast to this an irradiation under the same conditions except that the P D was 70 volts gave $\bar{k} = 0\ 172$ and one at 80 volts gave $\bar{k} = 0\ 302$ The amount of solution irradiated in these instances was 5 00 ml as in all former work   The proposed method of investigation was to observe the corresponding values of $\bar{k}$ obtained in irradiation of 10 ml quantities where a *screening* protective action would be made evident by a decreased mean speed coefficient   Three such observations gave a mean $k = 0\ 196$ with a d $= 0\ 008$

The screening protection here is obvious, but in order to obtain even stronger evidence of such protection we contrasted similarly results in which 5 and 10 ml of a solution of 0 07 per cent pancreatin were irradiated, obtaining, respectively, $\bar{k} = 0\ 156$ (a d $= 0\ 002$) and $\bar{k} = 0\ 0907$ (a d $= 0\ 0007$) for two instances of each

In order to ascertain whether the reduced speeds of reaction evi-

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[7] All amylase solutions were made to contain and diluted with solvent containing 30 ml per 100 of M/15 standard phosphate buffer mixture for pH = 6 7, as was the case already adopted for the substrate   Subsequent to this work an improved viscosimetric method was developed   Thompson, W R , Tennant, R , and Wies, C H , *J Biol Chem* , 1935, **108**, 85, Thompson, W R , *J Biol Chem* , 1935, **109**, 201

dent in the second 5 ml layer is due to a screening by the first 5 ml or to a divergence of the rays, two irradiations were performed in which 5 ml of the 0 02 per cent pancreatin plus 5 ml of the solvent[7] were irradiated together Thus all of the concentrations were approximately halved except those of the phosphate buffers, H^+ and OH^- The mean k so obtained was 0 257 (a d $= 0 018$), and a similar result with the 0 07 per cent pancreatin gave $k = 0 147$ These approximate the values obtained with 5 ml of the undiluted solutions which have been given (0 259 and 0 156, respectively)

That there is a definite protective effect due to screening is obvious Moreover, we may outline a simple theory of homogeneous absorption which gives close approximation with the observed results

Accordingly, we assume that (1) holds in any volume element wherein the effective radiation intensity is constant, that the rays are parallel, and, therefore, that a coaxial disk volume element of the cylinder of thickness, ds, approaches the condition of equal radiation intensity throughout its volume as a limit as ds approaches zero Furthermore, we assume that stirring is sufficient to maintain the concentration of active enzyme, Q, the same throughout the entire liquid mass at any instant of time If k is the mean speed coefficient in the infinitesimal layer of thickness, ds, the bottom of which is at a distance, s, above that of the whole cylindrical liquid system whose total altitude is s_a and mean speed coefficient is Λ_a, then, obviously,

$$(2) \qquad \Lambda_\alpha = \frac{\int_0^\alpha k\ ds}{s_\alpha}$$

So far nothing definite has been assumed about the function, k, but we shall assume that k is proportional to effective radiation intensity and that this varies in accord with a law of equal fractional absorption in traversing a given thickness of solution, $i\ c$

$$(3) \qquad k = k_0\ e^{-\lambda s},$$

where λ is the absorption coefficient for the solution In actual observations where non homogeneous radiation is involved a high relative absorption rate is generally found in the early layers, but (3) may approximately represent the facts, and the representation may be

close for large values of λs even if not throughout the whole range   In our ideal system we assume (3) to hold throughout   Then (2) and (3) give

$$(4) \qquad K_\alpha = \frac{\displaystyle\int_0^{s_\alpha} k_0\, e^{-\lambda s}\, ds}{s_\alpha} = \frac{k_0(1 - e^{-\lambda s_\alpha})}{\lambda\, s_\alpha}$$

Now, as in the case of 5 and 10 ml irradiations,

(5)   if $s_2 = 2s_1$, then

$$\frac{2K_2}{K_1} = \frac{1 - e^{-2\lambda s_1}}{1 - e^{-\lambda s_1}} = 1 + e^{-\lambda s_1},$$

whence, in this case,

$$(6) \qquad \lambda s_1 = \log_e \frac{K_1}{2K_2 - K_1}$$

Now, let a *prime* refer to the 0 02 per cent and a *double prime* refer to the 0 07 per cent pancreatin solutions, and assume that $\lambda'' = 3\,5\,\lambda'$ Then we may estimate $s_1\lambda'$ from the data on the 0 02 per cent solution by (6) and k_0 by substitution in (4)   From these values we may calculate the corresponding values of K_1'' and K_2'' which are given in the third column of Table I Similarly, we may estimate $s_1\lambda'$ and k_0 from the observed values for Solution 2 (0 07 per cent pancreatin) and calculate K_1' and K_2' as given in the fourth column   The observed values are entered in Column 1   The two sets of calculated values are obtained by regarding the data from one or the other of the two solutions (0 02 or 0 07 per cent) as perfect   A better result might be expected if we use instead a weighted mean of the two estimates of $s_1\lambda'$ and the mean value of k_0 obtained by substitution of this in (4) Thus we may give 3 5 times as much weight to the estimate of $s_1\lambda'$ from the data from the second solution as that from the first (roughly according to relative precision) and so obtain the values given in Column 2   These agree best with the observed values, and those by the extrapolation method in Column 3 worst as might be expected, but all indicate that the theory outlined above may serve at least as a first approximation

We have made a further study of the protective action of dog serum using two additional dogs In the former work it was merely demon-

strated that a protective action existed, but in this work we hoped to show something of the mode of action as in the case of the pancreatin solutions alone   Accordingly, 5 and 10 ml portions of solutions of pancreatin (0 02 per cent) containing 0 2 per cent of serum were irradiated with results given in Table II, and from these the respec-

TABLE I

Effects of Competitive Absorption upon the Speed Coefficient in Inactivation of Amylase with Ultraviolet Light

	Observed	Calculated (assuming $\lambda = 3.5\lambda$)		
		By weighted mean	From Solution 1	From Solution 2
k_0'		0 343	0 355	0 331
K_1'	0 259	0 263	0 259	0 264
K_2	0 196	0 208	0 196	0 211
K_1''	0 156	0 152	0 138	0 156
K_2''	0 0907	0 087	0 076	0 0907
λs_1		0 553	0 667	0 520

TABLE II

A Protective Action of Dog Serum (2 Parts per 1000) in Ultraviolet Irradiation of Pancreatin Solutions

No	Dog No.	Pancreatin	Q_0	$\dfrac{Q}{Q_0}$	k	v
		per cent				*ml*
18	2	0 02	10 07	0 627	0 0780	5
19	2	0 02	10 33	0 645	0 0489	10
20	3	0 02	9 61	0 452	0 132	5
21	3	0 02	9 98	0 462	0 0773	10
22	3	0 02	9 42	0 278	0 0711	10
23	3	0 02	10 12	0 305	0 119	5

tive values for λs_1 were 1 37 and 1 70 and for k_0 were 0 107 and 0 261 That some of the protection is due to screening is obvious in the table, but if the theory applied to the pancreatin solutions above held here also, then we should expect the same value for k_0, which is not the case However, this does not mean that we must account for some of the protection upon some other basis than screening, but indicates at

least that if screening alone is responsible then the *screening* or *filtering* properties of the system are not uniform but greater in the lower layers If all substances were in solution this is almost inconceivable However, it was due to the accumulation of a sediment in these solutions that experiments with any given preparation could not be prolonged, particularly in the case of Dog 2, and it is here that the lowest value of k_0 was encountered Furthermore, a slight drift toward increasing protection with time is apparent in the case of Dog 3 Accordingly, it is reasonable to assume that we had somewhat greater screening protection in the lower layers, possibly sufficient to account for the low k_0 observed The protective action of the serum of these two dogs was roughly the same as in the instance previously reported

It is obvious in (4) that $2K_2 - K_1$ is the mean speed coefficient for the *second layer* (upper half of the irradiated system), and that if we could measure this directly we should obtain more reliable results Thus a system such that the two *layers* could not mix would be more advantageous, or we might use our 5 ml system in the quartz tube as an instrument for measuring the effective intensity of radiations not absorbed by an interposed layer of solution of known thickness The superiority of such a method over others such as the employment of a thermopile is obvious as is also the futility of dependence upon absorption spectra as a means of detecting the kind of radiations responsible for a given chemical reaction unless that reaction alone utilizes energy from this source

CONCLUSION

Evidence has been presented which indicates that the protective action of dogs' sera in irradiation of pancreatin solutions with ultraviolet light is the result of a competitive absorption (screening action) A similar effect is found in simple pancreatin solutions for which we may account (at least to a first approximation) on the basis of assumed homogeneous absorption by a strong competitor in the solution for the radiations having inactivating power These observations are of interest in connection with the theory of what has been called *negative catalysis* especially in view of the marked effects of small quantities of the protecting substances

Taylor, H S, *J Phys Chem* 1923, 27, 322
Christiansen J A, *J Phys Chem*, 1924, 28, 145

RESTORATION OF THE POTASSIUM EFFECT BY MEANS OF ACTION CURRENTS

By W J V OSTERHOUT and S E HILL

(From the Laboratories of The Rockefeller Institute for Medical Research)

By the potassium effect we mean the P D observed in leading off from a region of *Nitella* in contact with 0 01 M KCl to one in contact with 0 01 M NaCl This may amount to 80 mv or more (potassium being negative in the external circuit)

This disappears when the cells are exposed to distilled water [1] This has been explained by saying that distilled water removes from the protoplasmic surface a substance,[2] *R*, which is produced in the cell and which moves into the surface It is dissolved out of the surface by distilled water and in consequence the surface ceases to distinguish between sodium and potassium so that the potassium effect is lost

We have performed experiments which indicate that *R* moves into the surface during the flow of the action current The nature of the experiments[3] may be illustrated by a typical example Single cells were prepared by cutting away the neighboring cells and were kept for several days in a nutrient solution (Solution A[4]) and then transferred to distilled water A cell which had been kept for 2 days in distilled water was arranged as in Fig 1 (*F* was killed by chloroform before the start of the record to give monophasic recording) The

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[1] Osterhout W J V and Hill, S E , *J Gen Physiol* 1933–34 **17**, 87, 99 105

[2] For convenience we speak of a single substance although it is highly probable that more than one substance is involved

[3] Unless otherwise stated the technique is that of previous papers *Cf* Osterhout W J V and Harris, E S *J Gen Physiol* 1927–28, **11**, 391 Osterhout W J V, and Hill S E *J Gen Physiol*, 1929–30 **13**, 547 1930–31, **14**, 385 473 1933–34 **17**, 87, Blinks L R , *J Gen Physiol* 1929–30 **13**, 361 The experiments were performed on *Nitella flexilis* Ag at a temperature of about 22 C

[4] For the composition of this solution see Osterhout, W J V and Hill S E *J Gen Physiol* , 1933–34 **17**, 87

record of C (upper curve) and D (lower curve) is shown in Fig 2 (E was not recorded)   At the start all contacts were 0 01 M NaCl

By means of a flowing contact[5] 0 01 M KCl was substituted for 0 01 M NaCl at D at the point marked 1 on the record   This produced no effect   An electrical stimulus (500 mv D C, giving an outgoing current at B) was then applied (at the point marked 2) and produced an action current at C followed by one at D Since D recovered it is evident that the potassium effect had not been restored by the action current   in other words it behaved as though it were in contact with NaCl   Two subsequent stimulations (at 3 and 4) had no effect

The next stimulation (at 5) produced very interesting results There was an action current with recovery at C (which was in contact
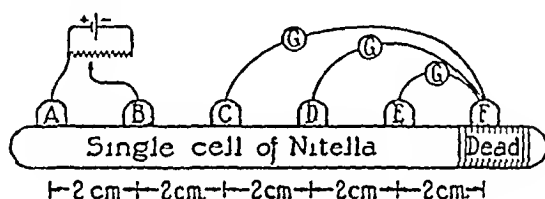
Fig 1   Arrangement for testing *Nitella* cells   GGG represent string galvanometers with vacuum tube amplifiers, arranged essentially as short period electrostatic voltmeters   For details see Osterhout, W J V, and Hill, S E, *J Gen Physiol*, 1933–34, 17, 87   Absorbent cotton, moistened with the contact solution, connected A, B, and F to saturated calomel electrodes, contacts C and D were flowing contacts (cf Osterhout, W J V, and Harris, E S, *J Gen Physiol*, 1927–28, 11, 391)   Between contacts the cell was surrounded by moist air

with 0 01 M NaCl) but at D, which was in contact with 0 01 M KCl, there was no recovery   We interpret this to mean that sufficient R was carried into the surface by the action current (helped, perhaps, by the preceding action current) to restore the potassium effect   When this is present 0 01 M KCl usually reduces the P D to a low value, as is evident here where the P D falls 100 mv

Since C had also been subjected to action currents it was of interest to see whether it had also acquired the potassium effect   On applying 0 01 M KCl to C, at the point marked 6, no potassium effect was secured   but when stimulated (at 7) a potassium effect appeared

5 Osterhout, W J V, and Harris, E S, *J Gen Physiol*, 1927–28, 11, 391

It is evident that as the result of the action currents at C and D the potassium effect was restored at both places   This effect can persist for some time as is evident from the record   When 0 01 M NaCl was applied to C and D (at 8) the P D returned to about the same point as at the start (about 89 mv positive for C and 100 mv for D)   Then 0 01 M KCl applied at D (9) and at C (10) produced a potassium effect,[6] the application of 0 01 M NaCl restored the positive P D at both places, showing that they were not injured

In other experiments it was found that after one or more action currents at a spot in contact with 0 01 M NaCl the potassium effect had been restored   This was shown by substituting 0 01 M KCl for 0 01 M NaCl   this produced a potassium effect at once (i e it was not necessary to stimulate again electrically after the application of 0 01 M KCl)

Such experiments have been performed in a variety of ways but always with the same general result   If the cells have been too long in distilled water they lose their irritability as well as the potassium effect and cannot be used for such experiments   Similar results have been obtained with cells leached in 0 0001 M HCl and 0 001 M NaOH [7]

It frequently happens that the first action current restores the po tassium effect   This finds a parallel in the experiments of Blinks[8] on *Chara* which he explains as the result of changes in pH   Such changes may, of course, play a role in *Valella*[9] but to what extent must be left to future investigation to decide

DISCUSSION

During the outward flow of the action current cations move from the sap and protoplasm into the outer protoplasmic surface and the fact that R does likewise might be thought to indicate that it is an organic cation   This idea suggested a trial of NH_3 NH_4^+, and

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[6] The response is delayed somewhat as often happens with the potassium effect Osterhout W J V and Hill S E *J Gen Physiol* 1933–34 **17**, 99

[8] Blinks L R *Proc Soc Exp Biol and Med* 1932–33 **30**, 156

[9] For example if R bears a positive electric charge and so moves outward during the action current a change in pH may affect the number of such cations espe cially if R is an ampholyte
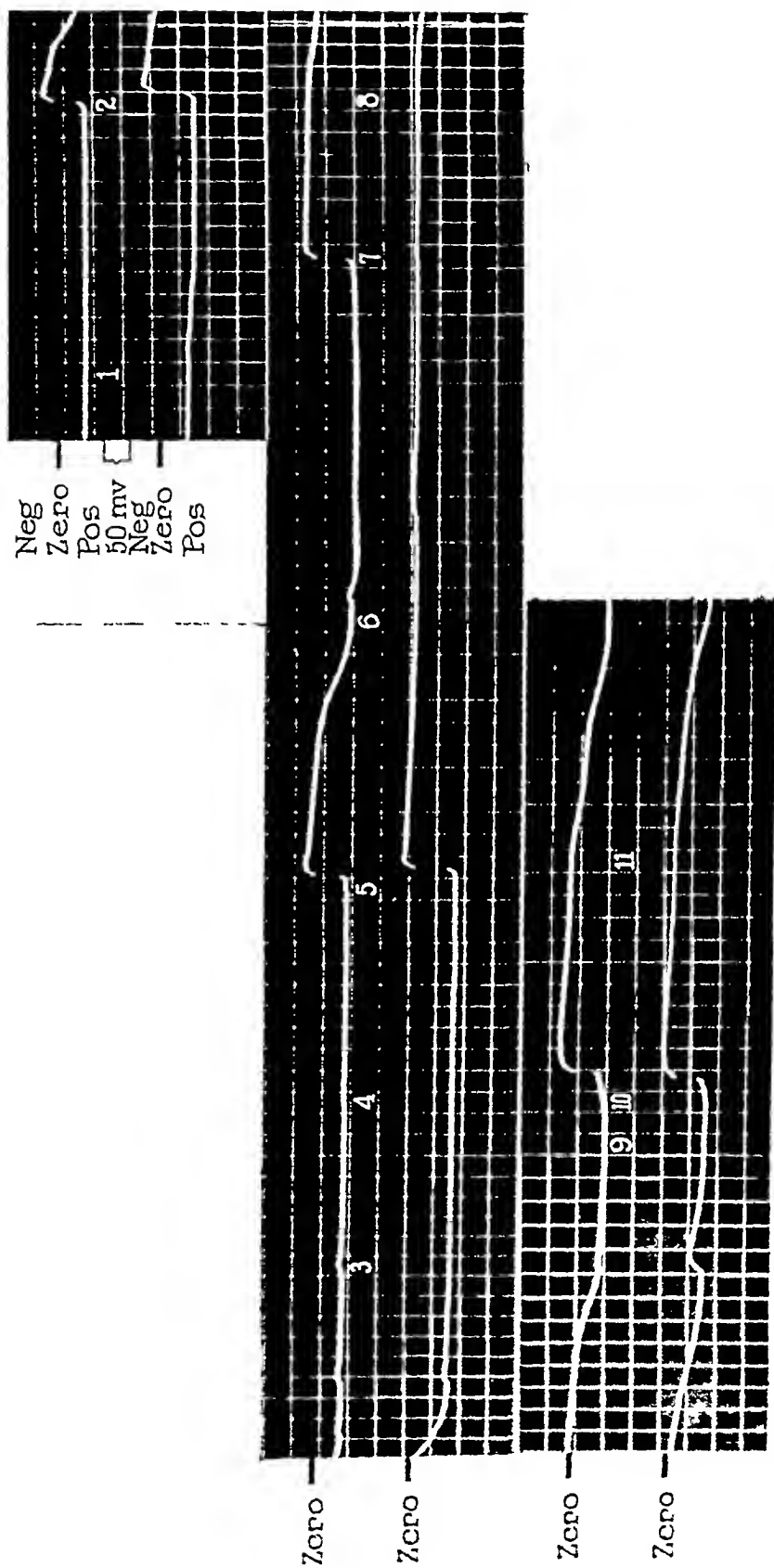
Fig 2

tetraethyl ammonium ion, all of which proved capable of restoring the potassium effect [10]

It must be remembered, however, that the action current is probably accompanied by a breakdown of the inner protoplasmic surface[11] which might permit the outward passage of R even though it bore no electrical charge   This question must be left to future investigation

The experiments are of considerable interest as indicating that action currents may have after effects which can be detected only by special means, e g in the presence of potassium but not in the presence of sodium   For if in the experiments described above only sodium had been present the changes would not have been perceived

This suggests that the after effects of action currents may be important in other cases, e g in "warming up" in athletic contests or in

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FIG 2 Photographic record (of a cell arranged as in Fig 1 with E omitted) showing the P D at C (upper curve) and D (lower curve)   The record starts with 0 01 M NaCl at C and D (before the start of the record Γ was treated with chloroform, reducing its P.D to a low and approximately constant value)

At the point marked 1 on the record, 0 01 M KCl was substituted for 0 01 M NaCl at D   This produced no change (i e no potassium effect) at 2, electric stimulation at B produced action currents at C and D, C recovered (because it was in contact with 0 01 M NaCl), and D also showed recovery, although in contact with 0 01 M KCl, because the potassium effect was not restored by the action current   Stimulation at 3 and 4 had no effect but at 5 it produced an action current at C and D   C recovered because it was in contact with 0 01 M NaCl, but there was no recovery at D (which was in contact with 0 01 M KCl) because the potassium effect was restored and in consequence the P D became 102 mv more negative   (The action current goes above zero on account of some positivity at Γ)

The application of 0 01 M KCl to C (at 6) had no effect but electrical stimulation at 7 produced an action current which restored the potassium effect at C (making the P D 100 mv more negative)   At 8, application of 0 01 M NaCl restored the positive P D at C and D and application of 0 01 M KCl at D (9) and at C (10) produced a potassium effect   Then 0 01 M NaCl was applied (11) at both places, restoring the positive P D and showing that no injury had occurred

The vertical lines are 5 seconds apart.   Temperature about 22°C

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[10] These results will be described in subsequent papers   Cf Osterhout, W J V and Hill, S E , Proc Soc Exp Biol and Med , 1934–35, 32, 715

[11] Osterhout, W J V , J Gen Physiol 1934–35, 18, 215

memorizing lists of words, where there is a gradual improvement in the efficiency of the performance

When the potassium effect has not been restored we should expect an action curve with a single peak   This is actually observed

SUMMARY

Treatment with distilled water removes from *Nitella* the ability to give the large potential difference between 0 01 M KCl and 0 01 M NaCl which is known as the potassium effect   The potassium effect may be restored by action currents   This might be explained by saying that distilled water removes from the surface a substance, R, which is responsible for the potassium effect and which moves into the surface during the action current and thereby restores the potassium effect

MECHANICAL RESTORATION OF IRRITABILITY AND OF THE POTASSIUM EFFECT

BY S E HILL AND W J V OSTFRHOUT

(*From the Laboratories of The Rockefeller Institute for Medical Research*)

Nitella exposed to distilled water loses its ability to propagate negative variations and to give the normal potassium effect[1] (*i e* the action of KCl in making the P D more negative) Apparently this is because the distilled water dissolves out of the protoplasm something which may for convenience be called R

Under normal conditions this substance presumably moves into the protoplasm from the sap fast enough to offset the dissolving action of the external solution But in distilled water the dissolving action is sufficiently accelerated to produce a deficiency of R

It occurred to us that if we could force sap into the protoplasm mechanically, by a pinch, we might succeed in restoring R sufficiently to bring back the normal behavior We have therefore made experiments of this sort

In some cases the result is very dramatic Cells which are unable to give any response to electrical stimulation may after a single pinch respond normally This result cannot always be depended on for if the pinch is too gentle several may be required to produce the desired effect and if it is too vigorous injury may occur The results, however, seem to show clearly that the normal behavior may be restored by forcing sap into the protoplasm

The procedure may be illustrated by citing some typical experiments These were performed on *Nitella flexilis* Ag at a temperature of 20–22°C

[1] Osterhout W J V, and Hill, S E *J Gen Physiol* 1933–34 17, 87, 99 105

[2] Unless otherwise stated the technique is that described in previous papers (*cf* Osterhout W J V, and Harris E S *J Gen Physiol* 1927–28 11, 391 Osterhout W J V and Hill S E *J Gen Physiol*, 1929–30 13, 547 1930–31 14, 385, 473 1933–34, 17, 87 Blinks L R. *J Gen Physiol*, 1929–30 13, 361)

Cells which had been freed from neighboring cells were left for several days in a nutrient solution (Solution A[3]) and then kept in distilled water until they lost their irritability and potassium effect (2 days or more)

An experiment with such a cell (arranged as in Fig 1) is shown in Fig 2   An electrical stimulus produced no effect since the irritability had been lost, but a pinch produced a mechanical negative variation As shown in previous papers,[4] this is due to a compression wave pro-
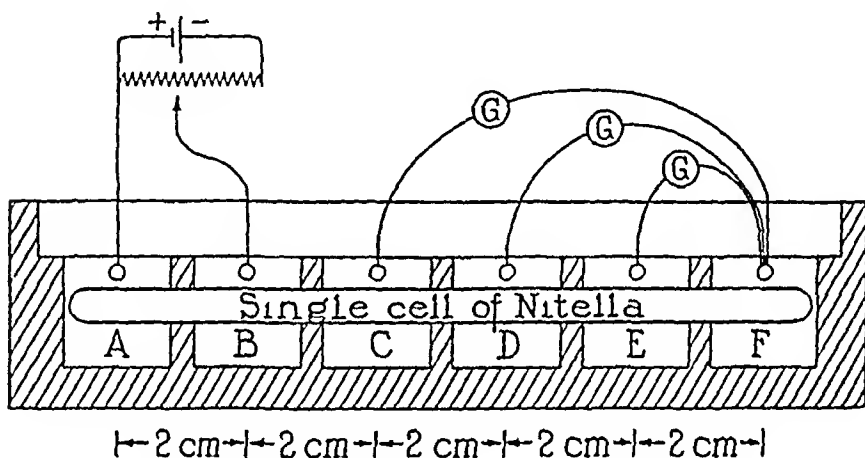
FIG 1   Arrangement for testing *Nitella* cells   *GGG* represent string galvanometers (3 strings inserted in a single magnetic field of a Type A Cambridge string galvanometer) with vacuum tube amplifiers, arranged as short period voltmeters   Silver-silver chloride electrodes dip in each cup   The *Nitella* cell passes through all of the cups (*cf* Osterhout, W J V, and Hill, S E, *J Gen Physiol*, 1933-34, **17**, 87)   Cup *D* had an overflow pipe enabling us to change the solution without interrupting the record by pouring the new solution into the cup

duced by the pinch   The compression wave travels along the cell, temporarily abolishing the outwardly directed P D at each point it

[3] For the composition of this solution see Osterhout, W J V, and Hill, S E, *J Gen Physiol*, 1933-34, **17**, 87

[4] The pinch sets up a compression wave which travels rapidly along the turgid cell the sap of which has an osmotic pressure about 4 atmospheres greater than that of the surrounding solution   *Cf* Osterhout, W J V, and Harris, E S, *J Gen Physiol*, 1928-29, **12**, 167, 355, Osterhout, W J V, and Hill, S E, *J Gen Physiol*, 1930-31, **14**, 385, 473

Fig 2 Photographic record showing the restoration of irritability by a mechanical stimulus   The cell was arranged as in Fig 1   It had been 2 days in distilled water and in consequence had lost its irritability   All contacts were 0 01 M NaCl

The top curve shows the p d between C and Γ the middle curve that between D and Γ and the bottom curve that between E and Γ   This produced no effect (the small temporary rise in the curves is due to spread of current from B)

At the place marked 1 on the record an electrical stimulus (300 mv in C) was applied giving an outgoing current at B

At 2 the cell was punched between L and F producing mechanical negative variations at all points   The beginning of the response at Γ is shown by the simultaneous downward movement at C D and E   The remaining portions of the curves are due in part to movement at Γ and in part to movements at the other spots   The responses at 3 and 4 are due in part to movement to the punch   The responses at 3 and 4 are

At 3 4 and 5 electrical stimuli produced responses showing that irritability had been restored by the punch

decidedly diphasic that it > is less so

The vertical lines are > seconds apart   Temperature about 22 C
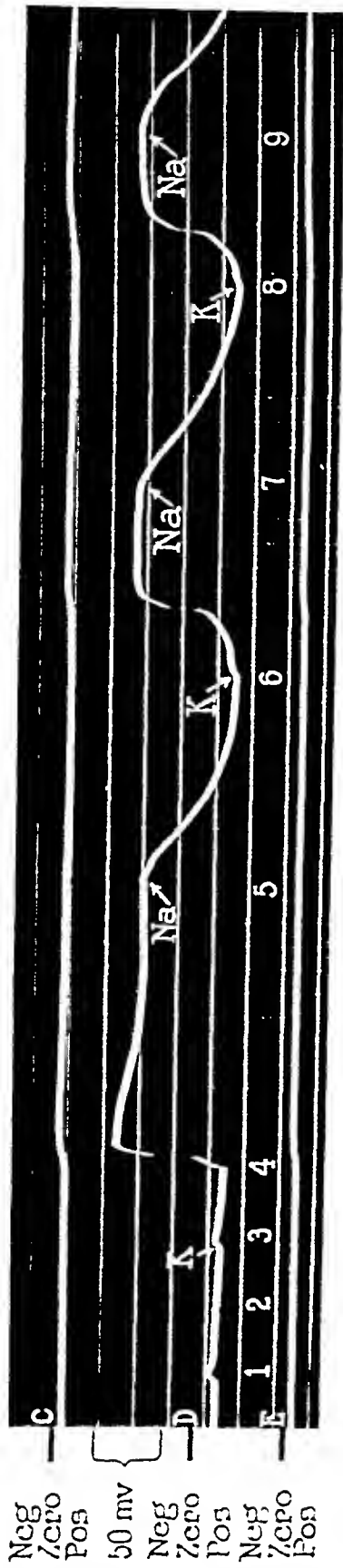
689

Neg
Zero
Pos

50 mv

Neg
Zero
Pos

Neg
Zero
Pos

Fig 3 Photographic record showing the restoration of the potassium effect by a mechanical stimulus. The cell was arranged as in Fig 1. It had been 8 days in distilled water and in consequence had lost both irritability, and the potassium effect. All contacts at the start were 0.01 M NaCl.

The top curve shows the p.d. between C and I, the middle curve that between D and F, and the bottom curve that between E and F.

At the place marked 1 on the record 0.01 M KCl was substituted for 0.01 M NaCl at D. This had practically no effect, at 2 this was replaced by 0.01 M KCl, again with practically no effect.

At 4 the cell was gently pinched near D and D became 83 mv more negative. There was no recovery because the potassium effect had been restored by the pinch (the level of the curve fell off a little and then became stationary). Then 0.01 M NaCl was applied (5) and brought the curve back to the former level. (The pinch produced no effect at C, E, or F and in consequence the movement of the middle curve is due wholly to changes at D. This is not surprising in view of previous experiments which show that the effect of a pinch dies out as the distance from the pinch increases.)

At 6, 0.01 M KCl was again applied at D, making the p.d. 75 mv more negative. the p.d. was restored by applying (7) 0.01 M NaCl. At 8, 0.01 M KCl was again applied followed by 0.01 M NaCl at 9. The vertical marks are 5 seconds apart. Temperature about 21°C
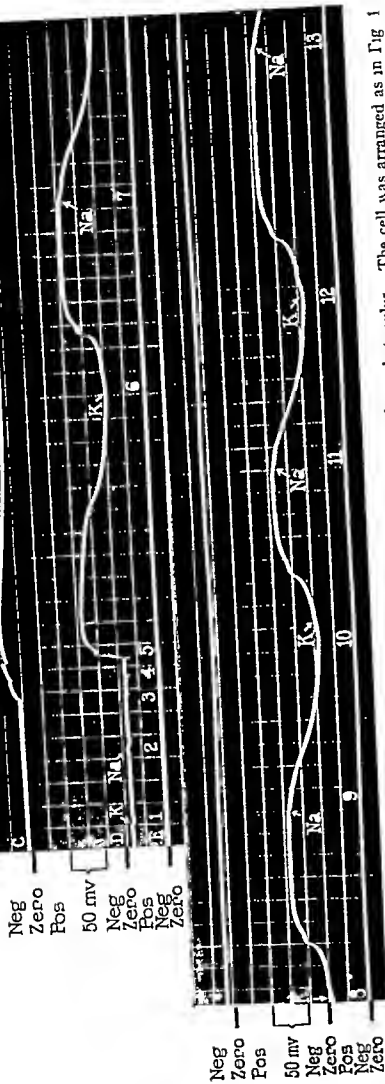
Fig 4 1 Photographic record showing the restoration of the potassium effect by a mechanical stimulus   The cell was arranged as in Fig 1
It had been 7 days in distilled water and in consequence had lost its irritability and the potassium effect   All contacts at the start were
0 01 M NaCl

The top curve shows the p d between C and F the middle curve that between D and F and the bottom curve that between E and F
At the place marked 1 on the record 0 01 M KCl was substituted for 0 01 M NaCl at D   this had practically no effect at 2 0 01 M NaCl was
applied in place of 0 01 M KCl

At 3 and 4 the cell was gently pinched between C and D but with no effect (except for a small change at C) but at 5 a stronger pinch
caused a mechanical negative variation at D from which it recovered since it was in contact with 0 01 M NaCl

At 6 (1 minute later) 0 01 M KCl was applied at D and D became 50 mv more negative   There was no recovery because the potassium effect
had been restored by the pinch but when 0 01 M NaCl was applied at 7 the curve returned to the level at the start of the experiment

At 8 0 01 M KCl was applied making the p d 50 mv more negative but when 0 01 M NaCl was applied at 9 the curve returned to the former
level This procedure was then repeated three times (to save space the final drop due to NaCl is not completed on the record)

The vertical marks are 5 seconds apart   Temperature about 22°C

691

touches   It probably does this by rupturing the protoplasmic sur-
faces, the rupture being immediately repaired

The pinch restored the irritability presumably by forcing sap into
the protoplasm   The restoration of irritability is clearly shown by
the fact that after the pinch electrical stimulation produced a re-
sponse in every case

Can the potassium effect also be restored in the same way?   To
test this an experiment was made as shown in Fig 3   The substitu-
tion of KCl for NaCl at D had no effect because the cell had lost its
potassium effect but when the cell was pinched so as to produce a
mechanical negative variation at D there was no recovery because the
pinch restored the potassium effect and in consequence KCl reduced
the P D to a low value   There was no injury as shown by the fact
that when NaCl was applied the P D returned to the normal value

The effect of the pinch in restoring the potassium effect does not
disappear at once for subsequent applications of KCl gave typical
potassium effects

A different type of experiment is shown in Fig 4   Here the cell was
pinched with NaCl in contact with D in consequence there was a
mechanical negative variation with recovery at D A minute later
KCl was applied at D and produced a potassium effect, but there was
no injury as shown by the application of NaCl which restored the
normal P D   Subsequent applications of KCl showed that the effect
persisted

DISCUSSION

It would seem that when sap is forced into the protoplasm by the
pinch,[4] it carries something which is responsible for the potassium
effect and for normal irritability   Although for convenience we speak
of this as R it is probable that it contains more than one substance
since we find, in some cases at least, that when cells are leached with
distilled water the potassium effect disappears before the irritability
is lost [5]   This is in harmony with the fact that in *Chara coronata* [6] [7]

action currents regularly occur although the potassium effect is normally lacking

A single pinch may produce only a partial restoration of the potassium effect   In such cases additional pinches may produce more effect but it is possible to overdo the matter and injure the cell (considerable variation exists in this respect)

Let us now consider the rôle of the two protoplasmic surfaces   When we substitute KCl for NaCl the change in P D (if it occurs at all) usually takes place within a few seconds which is presumably too short a time for potassium to penetrate through the protoplasm to the inner surface   We therefore suppose that only the outer surface is involved which presumably is not normally in contact with much potassium (either in the external solution or in the protoplasm)

The situation is quite different with the inner surface which is in contact with sap containing about 0 05 M KCl (and 0 05 M NaCl) Since there is presumably not much potassium in the protoplasm there is a marked potassium gradient across the inner protoplasmic surface and we suppose this to be responsible for the outwardly directed P D which is characteristic.

The treatment with distilled water which removes the potassium effect from the outer surface does not appear to affect the inner surface in the same way for its outwardly directed P.D seems not to be lessened   But the inner layer may be affected in such fashion as to prevent the increase of permeability which is necessary for the action current [6]   This would explain why a spot[7] treated with distilled water can act as a block while retaining its outwardly directed potential (100 to 300 mv)

We therefore suppose that the pinch affects both the inner and outer protoplasmic surfaces   Whether this effect depends wholly on the outward movement of R must be left to future investigation   In the case of the inner protoplasmic surface (which is in contact with the sap) the effect of the movement of R would seem to depend on forcing R into the aqueous layer of protoplasm[8] which lies between the two non-

[7] Osterhout, W J V , and Hill, S E , *J Gen Physiol* 1933-34 **17,** 87

[8] Even if R is produced in this layer it is presumably leached out by the action of the distilled water

aqueous protoplasmic surfaces, and so getting R on both sides of the inner surface layer   Presumably this layer is temporarily ruptured by the pinch[4] thus allowing R and K^+ to pass through after which the rupture is repaired   The outer layer may also be ruptured but this is less likely to happen because it is protected by being adherent to the solid cellulose wall

SUMMARY

Treatment of *Nitella* with distilled water apparently removes from the cell something which is responsible for the normal irritability and the potassium effect, (*i e* the large P D between a spot in contact with 0 01 M KCl and one in contact with 0 01 M NaCl)   Presumably this substance (called R) is partially removed from the protoplasm by the distilled water   When this has happened a pinch which forces sap out into the protoplasm can restore its normal behavior

The treatment with distilled water which removes the potassium effect from the outer protoplasmic surface does not seem to affect the inner protoplasmic surface in the same way since the latter retains the outwardly directed potential which is apparently due to the potassium in the sap   But the inner surface appears to be affected in such fashion as to prevent the increase in its permeability which is necessary for the production of an action current   The pinch restores its normal behavior, presumably by forcing R from the sap into the protoplasm

STUDIES ON THE ELIMINATION OF DYES IN THE GASTRIC AND PANCREATIC SECRETIONS, AND INFERENCES THEREFROM CONCERNING THE MECHANISMS OF SECRETION OF ACID AND BASE

By RAYMOND C. INGRAHAM and MAURICE B. VISSCHER

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INTRODUCTION

The mechanism by which secreting structures are able to concentrate certain constituents and dilute others presents interesting problems, particularly in such secretions as gastric juice, in which the concentration of one constituent, namely, the hydrogen ion, is a million times as great as in the fluid from which it came. The converse problem, dealing with the concentration mechanism of an alkaline secretion, is met with in the formation of the pancreatic juice. Numerous pertinent observations on the conditions and characteristics of these secretions have been made, some of which will be referred to in the course of the discussion of the results obtained in this investigation. Comprehensive reviews of the major problems discussed in this paper may be found in the monographs by Gellhorn (1929) and Babkin (1928), and in a shorter review by Carlson (1923). A thorough review of previous contributions to the problem will therefore be omitted and the reasoning underlying the present method of attack stated briefly.

Inasmuch as the stomach and pancreas appear to present two mechanisms of essentially opposite character, one structure serving to elaborate a highly acid secretion, the other a highly alkaline one, it seemed that a simultaneous study of the effects of various agents and conditions upon the activities of the two types of glands should yield valuable information concerning the mechanism of activity of each. In line with this thought, it would seem that a careful classification of those substances which appear in gastric and pancreatic juice respec-

tively when they are present in the circulating blood of the animal, might yield important clues as to the characteristics of the structures in the glands themselves responsible for secretion Experiments designed to yield this information have, therefore, been carried out The present report deals with the physicochemical characteristics of the dyestuffs which are eliminated by the stomach or pancreas, or both, after intravenous injection, and deductions from these observations concerning the mechanism of acid and alkali secretion

Numerous investigators have previously noted that certain dyes introduced into the blood appear in one or another glandular secretion Valuable comprehensive studies on a large list of dyes have been made by Kobayashi (1926), Dawson and Ivy (1925), and Crandall, Oldberg, and Ivy (1929) The first mentioned investigator confined his attentions to the appearance of dyes in the gastric juice, and found that among the dyes he studied all of those appearing in gastric juice were basic dyes, according to the usual classification He showed, furthermore, that the concentration of the dyes in the juice roughly followed the changes in hydrochloric acid concentration He inferred from his observations that the dye elimination and the hydrochloric acid secretion were intimately related Dawson and Ivy, however, found, besides numerous basic dyes, one ordinarily classified acid dye appearing in gastric juice After their exhaustive studies, they came to the conclusion that no known physical or chemical characteristic distinguished those dyes which did, from those that did not, appear in gastric juice after administration In a similar way, with regard to the pancreas, Crandall, Oldberg, and Ivy found both acid and basic dyes as classified by the ordinary technical dye application test appearing in pancreatic juice These investigators arrived at a similar conclusion, therefore, regarding the pancreas, since they were able to find no other physical or chemical characteristic which differentiates dyes which did, from those which did not, appear in its secretion

In view of the fact that many dyestuffs contain acid and basic radicals in such proportion that they might easily be expected to behave as amphoteric electrolytes at the hydrogen ion concentration of blood plasma, it seemed that a reinvestigation of the dyestuffs secreted with this in view, should be undertaken Similarly, since

many dyes are reversibly reduced in the body, and since the electron shifts involved in reduction or oxidation might alter the net charge or the dissociation characteristics of the dyes so that the chromogen might appear in the cation in the oxidized condition and in the anion when reduced, or *vice versa*, this characteristic of dyestuffs should also be taken into account in studies of their secretion

From the results that have been obtained it has been possible to sharply define dyes appearing in the gastric juice in one distinct electrochemical category and those appearing in the pancreatic juice in another

Methods

Dogs anesthetized with 50 mg chloralose and 250 mg urethane per kilo injected intravenously after initial etherization, have been used in this study It might be noted, in passing, that the barbituric acid derivatives, particularly amytal, have been found seriously to interfere with secretion in these studies, and their use was discontinued because of the high percentage of animals which failed to show adequate secretion with these anesthetics   The fundus was cannulated by tying a large glass tube into the pyloric canal   The esophagus was ligated to prevent contamination by saliva   The fundus was carefully washed with Ringer's solution and the secretion collected at intervals   The accessory pancreatic duct was cannulated and the pancreatic secretion collected continuously through a small bore rubber tube attached to the short glass cannula in the duct   Intravenous injections were made from a burette *via* a cannula in the femoral vein   Secretin of proven potency prepared by the method of Weaver, Luckhardt and Koch (1926) was injected intravenously to activate the pancreas and histamine hydrochloride was given subcutaneously, approximately 0 2 mg per kilo to bring about gastric secretion   The dyestuffs[1] under investigation were injected intravenously in doses of 20 mg per kilo dissolved in 100 cc of Ringer s fluid at body temperature   Ordinarily only one dye was tested on each preparation although in cases where no secretion of dye occurred a second dye has been introduced in the same preparation in order to obtain confirmatory information about particular dyestuffs without extending the series of animals required more than was necessary

The electrochemical characteristics of the dyes used have been studied by determining the direction in which the chromogen bearing ion migrates in the electric field

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The apparatus used for this purpose is shown in Fig 1   The vessels A and C are filled with a concentrated solution of sodium chloride   These vessels contain the electrodes which are connected to a 45 volt source of E M F   The vessel B contains a 0 05 per cent solution of the dyestuff in a buffer   The bridges D and E are prepared from bent glass tubing filled with a gel prepared from a 2 per cent solution of agar in normal potassium chloride

The solutions of the dyestuff were prepared with buffers at four different pH values, namely 1 2, 5 0, 7 3, and 8 4   A 0 05 per cent solution of the dye was used in all cases   If the dye was not soluble to that extent a saturated solution was used   Some dyestuffs were too insoluble to use at all at certain pH values Electrolysis was allowed to proceed for 1/2 hour   At the end of this time the bridges were examined and the direction and extent to which the dye had migrated was determined

The experiments on the dyes in the reduced condition were carried out only on those dyes which could be reversibly reduced
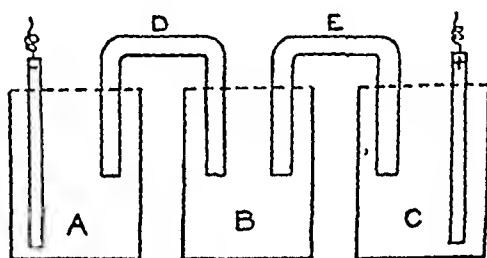
FIG 1   Diagram of apparatus for determination of charge upon dye chromogen

Reduction was accomplished by carefully adding a dilute solution of sodium hydrosulfite to the buffered solution of the dye   After reduction the surface of the solution was covered with a layer of mineral oil to exclude air   Dyestuffs which could not be completely reduced with sodium hydrosulfite were reduced with hydrogen and platinized asbestos   A small amount of sodium hydrosulfite was incorporated in the agar gel to remove dissolved oxygen   After electrolysis the agar plug was forced from the bridge and the color restored by placing the plug in 1 per cent hydrogen peroxide

The characteristics of the several dyes employed are indicated in the tables of results   The color index number is given as an aid in characterizing the dye

RESULTS

In Table I are listed those dyestuffs which are found to be eliminated in the gastric juice, classified as to their structure, their dye application class and as to the location of chromogen in cation or anion, according to the method described   It is to be observed that all of the

dyes found to be secreted by the stomach fall into the dye application class of basic substances, except fuchsin S It should be noted that the dye application classification is a technical one employed in the dyeing industry According to this classification, basic dyes are those which are salts of a color base and are substantive to cotton, while acid

TABLE I

Dyestuffs Eliminated by the Gastric Gland

Dye	Color index No	Chemical classification	Application class	Location of chromogen	Percentage of injected blood concentration appearing in secretion
Chrysoidine Y	33	Azo	Basic	In cation	75 0
Bismark brown	331	Dis azo	Basic	In cation	26 5
Fuchsin S	692	Triphenyl methane	Acid	Anion when oxidized both when reduced	0 5
Malachite green	657	Triphenyl methane	Basic	In cation	—*
Rhodamine B	749	Pyronine	Basic	Both anion and cation	6 1
Pyronine B	741	Pyronine	Basic	In cation	5 7
Acridine red	740	Pyronine	Basic	Both anion and cation	8 4
Indophenol	821	Indophenol	Basic	In cation	—
Methylene green	924	Thiazine	Basic	In cation	13 6
Methylene blue	922	Thiazine	Basic	In cation	15 6
Thionin	920	Thiazine	Basic	In cation	36 8
Toluidin blue	925	Thiazine	Basic	In cation	36 4
Azure II	923	Thiazine	Basic	In cation	—*
Nile blue B	913	Oxazine	Basic	In cation	0 8
Brilliant cresyl blue	877	Oxazine	Basic	In cation	11 3
Cresyl echtviolet	—	Oxazine	Basic	In cation	3 4
Neutral red	825	Azine	Basic	In cation	26 4
Neutral violet	—	Azine	Basic	In cation	27 2
Safranin O	841	Azine	Basic	Cation when oxidized both when reduced	27 3
Thioflavine T	815	Thiazol	Basic	In cation	1 3

* Not estimated.

dyes are salts of color acids and are substantive to wool in dilute sulfuric acid solution These criteria are obviously inadequate to distinguish amphoteric or partially amphoteric dyestuffs from those which ionize so that the chromogen is exclusively in one ion Although most dyes listed as appearing in the gastric juice are typically

basic, two of them, rhodamine B, and acridine red, show by the bridge experiments that the chromogen is present partially as anion and par-

TABLE II

Dyes Eliminated by the Pancreas

Dye	Color index No	Chemical classification	Application class	Location of chromogen	Percentage of injected blood concentration appearing in secretion
Resorcin green	1	Nitroso	Acid	In anion	5 10
Naphthol yellow	9	Nitro	Acid	In anion	0 18
Methyl orange	142	Azo	Acid	In anion	0 12
Orange I	150	Azo	Acid	In anion	0 43
Superchrome violet B	169	Azo	Acid	In anion	0 51
Wool blue CG	59	Azo	Acid	In anion	0 69
Fast fuchsin 6B	56	Azo	Acid	In anion	0 63
Methyl red	211	Azo	Acid	In anion	1 63
Alpha azurine 2G	712	Triphenyl-methane	Acid	In anion	0 18
Rosolic acid	724	Triphenyl-methane	Acid	In anion	0 23
Fuchsin S	692	Triphenyl-methane	Acid	Anion when oxidized, both when reduced	0 23
Gentian violet	680	Triphenyl-methane	Basic	Cation when oxidized, both when reduced	0 08
Fuchsin Y	677	Triphenyl-methane	Basic	Cation when oxidized, both when reduced	0 71
Uranine yellow	766	Pyronine	Acid	In anion	0 48
Acid-fast violet GRF	758	Pyronine	Acid	In anion	0 52
Eosin BS	771	Pyronine	Acid	In anion	—*
Phenol red	—	Pyronine	Acid	In anion	—*
Phenolphthalein	764	Pyronine	Acid	In anion	0 34
Cresol red	—	Pyronine	Acid	In anion	0 23
Acridine red	740	Pyronine	Basic	Both anion and cation	1 80
Rhodamine B	749	Pyronine	Basic	Both anion and cation	1 43
Brilliant blue CFC	855	Azine	Acid	In anion	0 43
Wool fast blue BL	833	Azine	Acid	In anion	0 05
Safranin O	841	Azine	Basic	Cation when oxidized, both when reduced	0 41
Indigo disulfonate	1180	Natural	Acid	In anion	1 00

* Not estimated

tially as cation, although the dyes are both classed as basic in the technical terminology Furthermore, three other dyes in the list

behave anomalously One, fuchsin S, ionizes with the chromogen in the anion when in a fully oxidized form, thus justifying its classification as an acid dye in that state, but shows migration of dye to both anode and cathode when reduced The other two which are typically basic in the oxidized form when reduced are likewise partially amphoteric

Similarly in the case of dyes found in the pancreatic juice after intravenous injection, as shown in Table II, the dyestuffs fall in both acid and basic classes, according to the usual technical dye application test When the location of the chromogen is studied by the bridge method, however, it is seen that in each of the four dyes, fuchsin Y, acridine red, rhodamine B, and safranin O, which are basic dyes according to the technical classification, the chromogen is in reality capable of being in either cation or anion

It is particularly significant, in connection with the fact that no typical basic dyes appear in pancreatic juice, that the dye secretion is being studied in these experiments simultaneously in stomach and pancreas, and that therefore the influence of the blood concentration of the dye is automatically controlled Since basic dyes leave the blood rapidly it might be urged that the pancreas could not for that reason secrete them This would be a valid argument except for the fact that at the same concentration in the blood the gastric glands are actually able to secrete them Obviously, therefore, there is a significant difference in the behavior of the two types of secreting structures toward these dyes

A good many dyes do not appear in either gastric or pancreatic juice after intravenous injection As seen in Table III, these dyes fall into a wide variety of chemical classifications It is not of particular importance to the theory that dissociation characteristics and charge determine which dyes can appear in the two juices, whether or not all acid dyes appear in pancreatic juice and all basic ones in gastric juice, the important point is that no electropositive dyes are found appearing in pancreatic juice, nor electronegative dyes in gastric juice It should be noted at this point that a good many dyes which Kobayashi, and Ivy and his collaborators, found not to be excreted, have been found in these studies to be eliminated either by the pancreas or stomach, as shown in the first two tables This difference in

observation is to be attributed to the facts that the earlier investigators overlooked certain dyes appearing in the reduced form, and that they employed smaller dosages of dye It has been found in these studies that certain dyes, such as fast wool blue B, which does not appear when administered at the standard dosage used in these experiments and is therefore not included in Table II, appear when the concentration in the blood is increased by administering double the

TABLE III

Dyestuffs Not Eliminated by Either Organ

Dye	Color index No	Chemical classification	Application class	Location of chromogen
Naphthol green	14	Nitroso	Acid	In anion
Fast wool blue B	209	Azo	Acid	In anion
Wool orange 2G	27	Azo	Acid	In anion
Wool scarlet BR	280	Azo	Acid	In anion
Trypan red	438	Dis azo	Acid	In anion
Trypan blue	477	Dis azo	Acid	In anion
Light green yellowish SF	670	Triphenylmethane	Acid	In anion
Water blue	706	Triphenylmethane	Acid	In anion
Brilliant green	662	Triphenylmethane	Basic	In cation
Methyl violet 2B	680	Triphenylmethane	Basic	In cation
Pyrrol blue	—	Triphenylmethane	Acid	In anion
Alizarin red S	1034	Oxyketone	Acid	In anion
Alizarin sapphire	1054	Oxyketone	Acid	In anion
Erythrosine B	773	Pyronine	Acid	In anion
Rose bengal	777	Pyronine	Acid	In anion
Brom phenol blue	—	Pyronine	Acid	In anion
Brom thymol blue	—	Pyronine	Acid	In anion
O-Cl-phenol indophenol	—	Indophenol	Acid	In anion
Thioflavine G	815	Thiazol	Acid	In anion
Cochineal powder	1239	Natural	Acid	In anion

dose No increase in dosage, however, has been found to cause completely acid dyes to appear in normal gastric juice, or completely basic ones to be secreted by the pancreas

In Table IV are presented in summary the characteristics of those seven dyestuffs which have been found to be anomalously secreted by the stomach or pancreas Excepting these seven substances, all the dyes eliminated by the stomach are typical basic dyes with the chromo-

gen in the cation, while all of the dyes eliminated by the pancreas are typical acid dyes with the chromogen in the anion   In the case of the six so called basic dyes appearing in the pancreatic juice, the two pyronines, acridine red and rhodamine B are amphoteric and are found to migrate partially to the anode and partially to the cathode between

TABLE IV

Anomalous Dyestuffs

Dye	Chemical class	Usual class fi cation	Location of chromogen	Elimination		Remarks
				Stomach	Pancreas	
Fuchsin S	Triphenyl methane	Acid	Anion when oxidized, both when reduced	+	+	Chromogen becomes partially amphoteric upon reduction
Fuchsin Y	Triphenyl methane	Basic	Cation when oxidized both when reduced	−	+	Chromogen becomes partially amphoteric upon reduction
Gentian violet	Triphenyl methane	Basic	Cation when oxidized, both when reduced	−	+	Chromogen becomes partially amphoteric upon reduction
Malachite green	Triphenyl methane	Basic	Cation when oxidized both when reduced	+	−	Chromogen becomes partially amphoteric upon reduction
Acridine red	Pyronine	Basic	Both in anion and cation	+	+	A partially amphoteric dye
Rhodamine B	Pyronine	Basic	Both in anion and cation	+	+	A partially amphoteric dye
Safranin O	Azine	Basic	Cation when oxidized both when reduced	+	+	Chromogen becomes partially amphoteric upon reduction

pH 1 2 and pH 8 4   They may be designated according to the classi fication of Bethe (1920) as partially amphoteric dyes   Their electro negativity increases in alkaline solution and their electropositivity increases in acid solution   The other three basic dyes in this group, fuchsin Y, gentian violet, and safranin O, show the chromogen ex

clusively in the cation in the ordinary oxidized condition, but become partially amphoteric on reduction

It is apparent that those dyes which are partially amphoteric would have opportunity to appear in both gastric and pancreatic juices, as they do, and that in the case of a dye whose chromogen changes its charge on reduction there is, likewise, the opportunity for appearance in either juice, depending upon the state of oxidation or reduction

In the case of the ordinarily classified acid dye, fuchsin S, there is some question as to whether it is actually secreted by the acid forming parietal cells or by mucus secreting cells   Five experiments were carried out using this dye   In three experiments the gastric juice was faintly tinted red and in two no coloration was noted   In the positive experiments large quantities of deeply stained mucus were found in the stomach   The low concentration of fuchsin S in the gastric juice is also significant since all other secreted dyes were present in high concentrations   It is true, however, that the behavior of fuchsin S upon reduction would explain its appearance in the gastric juice, but one must then assume that reduction takes place after the dye has passed from the blood into a more acid environment since at the pH of the blood fuchsin S is present as a colorless carbinol, incapable of being reduced

It is well known that the reduction of dyes in such a system as exists in the tissues is a relatively slow process (Voegtlin, Johnson, and Dyer, 1924) and that, therefore, the dyes which are capable of reversible reduction may logically be expected to be found partially in the oxidized and partially in the reduced form in the tissues for some time A further investigation is being continued on this point in order to determine, if possible, the oxidation-reduction characteristics of the gland cells themselves from observations on the state of oxidation or reduction of the dyes in the secretions   Preliminary observations seem to show that the gastric glands are strongly reducing as compared with the pancreas, inasmuch as certain dyes appear in gastric juice in the reduced form, while no dyes have appeared in pancreatic juice reduced   For example, pyronine B and indophenol appear in the gastric juice in the completely reduced form and can only be detected by the addition of an oxidizing agent

THEORETICAL DISCUSSION

Since it is found that those dyes whose chromogen is constantly present in the anion and are, therefore, electronegative are secreted only in the pancreatic juice, and that those dyes whose chromogen is constantly in the cation and are, therefore, electropositive are only secreted in the gastric juice, and since, further, all other dyes appear ing in either juice are found to be either partially amphoteric or to alter the charge on their chromogen upon reversible reduction, it appears that a generalization can be made, namely, that only dyes whose chromogen is electropositive under suitable conditions appear in gastric juice and only those dyes whose chromogen is electronegative under proper conditions appear in pancreatic juice   This uniformity in the group of more than sixty dyes studied in this way could scarcely be fortuitous   Since solubility, migration velocity, and other physical properties of dyes excepting their dissociation characteristics are not related to the sign of the charge, the conclusion seems proper that factors related to the character of the electric charge determine the conditions of passage of these substances through the glands   Two major alternatives suggest themselves in explanation of these findings They are based upon two quite different views as to the mechanism of penetration of substances through biological membranes   One is based upon the so called pore theory and the other upon the non aqueous phase solubility concept   Specific ion permeability according to the first mechanism is due to the existence of charges on the walls of membrane pores   According to the second view, transportation across membranes is not related to conditions of ionization in the membrane phase, but depends upon the partition coefficients for various substances between aqueous and non aqueous phases, the membrane being considered to consist of the latter—a completely continuous but perhaps extremely thin layer   In order to account for the selective permeability of specific ions by the membrane itself on this view it is possible to postulate acid and basic substances in this non aqueous phase, in which chemical combination may account for specific solubility   On the other hand the membrane might be quite non selective and the conditions of ionization of dyestuffs at various H^+ concentrations on either side of the membrane might account

for distribution differences   That this latter view does not provide a universally satisfactory explanation will be shown below

Two rather separate questions are involved in the problem at hand The method of restraint of entrance of the acid dyes by the gastric glands, and the basic ones by the pancreas, constitutes one problem, the mode of permeation of the opposite class in each case may be looked upon as quite a separate question   As will be developed more at length below, it is possible to interpret the restraint of one electro-chemical class of dyes as due to polar adsorption on oppositely charged surfaces   These surfaces may be the continuous phase of a colloidal gel of proper characteristics   A positive charge would thus restrain dye anions   Since dye cations appear in the secretion their passage must somehow be explained   Several suggestions as to possible mechanisms are given below, but it must be admitted at once that there is as yet insufficient information to permit of a positive conclusion

Dyestuffs are well known to be substances capable of strong polar adsorption, in which they differ strikingly from small ions such as sodium or chloride (Bogue (1924) and Kruyt (1927))   Since electronegative dye ions would be firmly bound to electropositive structures by polar adsorption and since they do not appear in gastric juice, it seems not unlikely that their failure to so appear is due to adsorption on electropositive structures

The relation of the charge on a membrane to the conditions of passage of dyes can be observed in the following simple experiment   Collodion membranes can be impregnated with benzoic acid and the electronegativity increased   To 10 per cent parlodion in equal parts of alcohol and ether was added benzoic acid up to 4 0 per cent   Membranes were cast as previously described   As indicated in Table V both acid and basic dyes dialyze through the membrane with low or zero benzoic acid content, but with 4 per cent of the latter, basic dyes are completely restrained, while the acid dye still passes through Since the benzoic acid increases the negative charge on the membrane it seems that polar adsorption accounts for the impermeability to positive dyes

The benzoic acid collodion membrane is partially impermeable to inorganic anions also, since with 0 001 N KCl on one side and 0 1 N

on the other an E M F can be measured with calomel electrodes, with the more concentrated side negative   The magnitude is only 10–20 per cent of the theoretical maximum, which one obtains with thoroughly dried membranes (Michaelis, 1925)   In spite of this partial impermeability to salt anions the dye anions are able to penetrate This may be due simply to the large size of the pores, or to other factors

The important result of this experiment is to show that a charged membrane can completely restrain passage of dyes of opposite charge

TABLE V

Passage of Dyestuffs through Collodion Membrane Containing Benzoic Acid

Dyestuff	Concentration of benzoic acid in collodion	Concentration of dye in dialyzate†
	per cent	per cent
Methylene blue	0 0	6 5
Fast fuchsin 6B	0 0	16 0
Methylene blue	2 0	3 0
Fast fuchsin 6B	2 0	16 0
Methylene blue	4 0	0 0
Fast fuchsin 6B	4 0	6 4

* The percentage of benzoic acid is based on the original collodion solution from which the membranes were cast

† The composition of the dye in the dialyzate at 2 hours time is expressed as per cent of the original dye solution

A similar result is obtained with formalinized gelatin membranes These membranes were made by pouring 20 per cent gelatin solution at about 70°C into test tubes, allowing complete drainage, fixing the gel by immersion in cold water, followed by treatment for 30 minutes with 15 per cent formalin   By placing these membranes in acid or alkaline solutions their charges were reversed, and their permeability characteristics noted   As indicated in Table VI, the acid dyes were restrained when the membrane was electropositive, that is, when the gelatin dissociated as a base, and vice versa on the alkaline side of the isoelectric point where it acted as an acid   The restraint of oppositely charged dyes was not absolutely complete in the case of these mem

branes, but the changes are sufficient to corroborate the previous conclusion that polar adsorption is responsible for the phenomenon Bethe (1922) found such adsorption in the case of parchment membranes

If these inferences can be transferred to the cases of the secreting structures of the gastric and pancreatic glands, it could be deduced that the membranes across which secretion occurs in the gastric glands are electropositive, while in the case of the pancreas they are negative

The passage of electropositive dyes across a positively charged membrane into the gastric juice offers numerous problems   A completely

TABLE VI

Dialysis of Dyes through Gelatin Membranes

Dye	Time of dialysis	Class	Concentration of dye in dialyzate*	
			In NaOH	In HCl
	hrs			
Alizarin sapphire	$3\frac{1}{2}$	Acid	1 0	0 008
Rhodamine B	2	Amphoteric	2 0	5 7
Methylene blue	2	Basic	1 0	8 4
Wool scarlet BR	$3\frac{1}{2}$	Acid	3 3	0 1
Fast fuchsin 6B	$3\frac{1}{2}$	Acid	4 9	0 1
Safranin O	$3\frac{1}{2}$	Basic	1 9	4 0
Neutral red	$3\frac{1}{2}$	Basic	Insoluble	3 9

* In per cent of concentration in original solution

cation-impermeable membrane should not allow any dye cations to pass through   It may be that the basic dyes do not pass through in a strongly ionized state   Many of these dyes are capable of reversible reduction and some, as noted above, actually appear in the juice in the leuco form   In this state the dyestuff has lost its strongly polar character because the imido-nitrogen is changed to amido, which has a very much lower dissociation constant   It is well known that weak bases such as ammonium hydroxide penetrate cell membranes readily while strong electrolytes such as sodium hydroxide permeate slowly if at all through the same type of membrane   Reduction of an acid dye causes no change in its polar group, consequently it would not behave similarly   Not all of the basic dyes appearing in gastric juice

are capable of reversible reduction   As a class the basic azo dyes are not.   It is significant that these dyes do not contain the imido nitrogen group, and therefore do not originally have the strongly polar character possessed by dyes containing this group

Penetration of certain dyes in reduced form was noted by Brooks (1926) in the case of *Valonia*   Wertheimer (1923) and Amberson and Klein (1928) have studied the frog's skin in relation to dye permeation and electromotive conditions   This membrane is selectively permeable to basic dyes in the outward direction, and to acid dyes in the opposite   The charge depends upon the pH and can be reversed on passing the isoelectric points which are different for the inner and outer layers of the skin

Several other hypotheses might be advanced to explain the mechanism of permeation of basic dyes into gastric juice   As mentioned above, it is unnecessary to assume passage in an aqueous phase   Osterhout (1933) has called attention to the possibility of explaining many perplexing permeability problems on the basis of non aqueous phase solubility   Irwin (1928) has developed a partition coefficient theory in this connection which would under certain conditions explain concentration of basic dyes in an acid secretion, and acid dyes in an alkaline secretion

If a non aqueous phase separates two aqueous phases, and if the dye is soluble in the non aqueous phase, the characteristics of the two aqueous phases will determine the distribution at equilibrium   In the case of an acid dye, $HD \rightleftharpoons \overset{+}{H} + \overset{-}{D}$, the dissociation constant will be $K_a = \dfrac{[\overset{+}{H}] \times [\overset{-}{D}]}{[HD]}$ If only HD permeates the non aqueous phase, its concentration should be identical in the two water systems at equilibrium   This is true since the distribution coefficient between the aqueous and the non aqueous phases for the case in question is $\dfrac{C(1-\alpha)}{C_1} = K_d$, where C is the total concentration of dye (dissociated and undissociated) in the water phase, C_1 the concentration in the non aqueous phase, and α the degree of electrolytic dissociation in water (cf Taylor, 1930)   The quantity of $\overset{-}{D}$ on either side will depend upon the $\overset{+}{H}$ concentration   If both aqueous phases are

strongly buffered and are at different acidities, the one with the higher $\overset{+}{H}$ concentration will accumulate little $\overset{-}{D}$ as compared with the other To take a numerical example let the pH of the blood be taken at 7 4 and that of gastric juice as 1 0   Since at equilibrium, if $[HD]$ is negligible,

$$\left[\overset{+}{H}\right]_b \times \left[\overset{-}{D}\right]_b = \left[\overset{+}{H}\right]_g \times \left[\overset{-}{D}\right]_g$$

$$\frac{\left[\overset{-}{D}\right]_b}{\left[\overset{-}{D}\right]_g} = \frac{\left[\overset{+}{H}\right]_g}{\left[\overset{+}{H}\right]_b} = \frac{10^{-1}}{10^{-7\,4}}$$

Thus the ratio of acid dye ion in blood to gastric juice would be about 1 0 000001   This small fraction of the amount in blood would be undetectable by our methods

This reasoning takes no account of the undissociated fraction, however, which in some instances is large, as in the case of the azo dyes If α is less than 0 9 the amount of undissociated dye at equilibrium would be amply sufficient to be detected by our methods   The degree of dissociation of many of the dyes used is much less than this figure at the pH values in question   We believe that this difficulty invalidates such an otherwise attractive hypothesis, since it will not account for all cases   The calculations for a specific case are given in Appendix I   It can be seen that at equilibrium there should be significant quantities of many acid dyes in the gastric juice, on this hypothesis Since they are not found, the theory, at least in its simplest form, must be abandoned   There remains, however, the possibility that the rate of passage of the two classes of dyes is different   Thus, acid dyes might pass much more slowly through the gastric gland membranes than do basic dyes   This would involve a problem in membrane permeability, however, and can be considered as a special aspect of the following case

The ability of a substance to penetrate a membrane can be considered to be dependent upon its solubility in the membrane, if the latter consists of a continuous phase   The pore concept, considered first, contemplates passage through an aqueous phase in a non-homogeneous membrane   If one supposes that solubility in a non-aqueous phase determines the conditions of passage of dyes, then there should

he some characteristic which dyes that penetrate have in common
Simple solubility in lipoid solvents does not provide such a common
characteristic (Ivy, Kobayashi) The possibility of compound forma-
tion would, however, provide a basis for separation between acid and
basic dyes If this were the mechanism, one would expect the gas
tric mucosa to accumulate within its secreting structures, the type
of dye which would penetrate into the gastric juice As a matter
of fact, the opposite is noted Dyes which do not appear in gastric
juice are accumulated in the gastric gland tissue Likewise in the
pancreas, basic dyes, which are not secreted by it, are accumulated
in its cells A serious objection to the validity of deductions from
these latter observations, is that one cannot be certain of the exact
histological structures responsible for secretion If only the cell
walls of parietal cells adjacent to the foveolar lumen are responsible
for determining the character of materials entering gastric juice,
then, of course, what materials accumulate in the rest of the cells is
of no consequence It is therefore not possible to rule out the con
cept of non aqueous phase solubility as the mechanism of selective
dye secretion, but it is not supported in any of our observations

Without insisting upon the finality of the interpretation it may
properly be pointed out that if the selective permeability be con
sidered as due to electrostatic charge characteristics, certain inter
esting deductions follow as to the mechanism of acid secretion in the
stomach, and alkali secretion in the pancreas According to the
pore theory if the secreting cell membrane in the gastric glands is
positively charged it would be impermeable to inorganic cations
Mond and Hoffmann (1928) have studied such membranes and found
them to behave so Such a membrane would under suitable con
ditions permit passage of chloride ions into a secretion while restrain
ing inorganic cations such as sodium

It is altogether likely that gastric juice is essentially an isotonic
solution of HCl Hollander (1934) has shown that when fundic
pouch secretion is studied the composition of the juice approaches a
pure aqueous solution of hydrochloric acid as the content of organic
solids present approaches zero Extrapolating to zero protein con
centration, he finds that what he calls the true parietal secretion
would actually be a solution of HCl isotonic with blood plasma Hori

(1933) has also shown that the difference between total and free acidity approaches zero, at zero protein content of juice   The fact that the parietal component of gastric juice is secreted as a virtually pure HCl solution would seem consistent with the view that chloride is able to appear in the juice because of selective anion permeability as suggested above

The origin of the hydrogen ion in such a system is not clear from the foregoing considerations   In a subsequent paper we will present model experiments in which it is possible to supply hydrogen ion for the chloride by a method of anion exchange   Carbon dioxide is made to combine with water on one side of a membrane impermeable to cations, the hydrogen ion resulting from dissociation remains, while the bicarbonate ion moves out of the compartment to maintain electrostatic neutrality   The work involved in the secretion of hydrochloric acid is not that of separating anion from cation, but simply that of substituting one cation for another under suitable circumstances   Jacobs and Parpart (1932) have considered this problem in connection with the chloride shift in the erythrocyte

Reference should be made to observations reported by Henning (1932) upon the living frog stomach, which are apparently in disagreement with the observations reported here   In a microscopic study Henning found that acid dyes strongly stained the fundus region   In this observation he confirms others already referred to It is obvious, however, that one should not confuse the ability of the cells to pick up the stain with their ability to secrete it   He did not collect gastric juice but tried to determine whether dyes were secreted or not by wiping off the mucous membrane of the whole stomach with pledgets of cotton   He found certain acid dyes appearing in the secretion in the stomach, and, in fact, made the generalization that only acid dyes are so secreted   He was entirely in error, however, in classifying magdala red, neutral red, and methylene blue as acid dyes, inasmuch as they are well known (Cain and Thorpe, 1918) to be basic   Furthermore, he is in complete disagreement with all other workers (Dawson and Ivy (1925), Kobayashi (1926), and ourselves) in finding the acid dyes, fluorescein, eosin, erythrosine, trypan blue, and Congo red secreted   He has no proof, however, that these dyes were secreted by the fundic glands, which the other workers

have been concerned with, and it therefore seems likely that they were actually secreted by the pyloric glands, which have an entirely different type of secretion   It is well known that the pyloric glands secrete a neutral or alkaline fluid (Babkin, 1928)   In view of the fact that acid dyes are readily secreted by the pancreas, the typical alkali secreting gland, it would not be surprising if the pyloric glands might do likewise   In this connection it should also be noted that the experiments reported in the present report refer to secretion under strong histamine stimulation   This excitant, as Vineberg and Bahkin (1931) have shown, calls forth the highly acid non mucous secretion from the stomach   Pilocarpine and certain types of vagal excitation, on the other hand, yield a secretion rich in organic components

With regard to the pancreatic secretion, a picture strictly analogous to that existing in the gastric glands can be drawn   Since dye cations are restrained, it may be supposed that the membrane possesses a negative charge   Inorganic anions would be restrained by electrostatic repulsion while the cations would pass through   In this respect the membrane would behave like the frog skin, in diffusion from without in, as studied by Wertheimer (1923)

CONCLUSIONS

1   All dyes appearing in gastric juice after intravenous injection in the dog are characterized by having their chromogen in the electropositive ion under suitable conditions

2   All dyes eliminated in pancreatic juice ionize with the chromogen electronegative under proper circumstances

3   The amphoteric characteristics of certain dyestuffs, as well as the changes in charge associated with reversible reduction in others, have been taken into consideration, and the lack of success of previous investigators in finding a common characteristic of dyes secreted by the gastric glands differentiating them from those secreted by the pancreas, has been shown to have been due to failure to take these potentialities of the dyestuffs into account

4   Several possible hypotheses concerning the mechanism of selectivity to dyestuffs have been considered   Differences in distribution in acid, neutral, and alkaline phases will not account for selective

secretion without postulating also specific membrane permeability It is pointed out that the theory most thoroughly in accord with all the facts observed is based upon the pore concept To restrain electronegative dyes by polar adsorption, the pores of the membranes of the gastric glands would have to be positively charged Such pores would constitute an electrostatic filter, restraining from passage all mobile ions of the same charge The anions, which in plasma are mostly chloride, could pass this barrier into the secretion In order to have hydrochloric acid formation, anion exchange would have to occur, bicarbonate, lactate, or some other anion from the gland lumen returning to balance chloride entering, leaving the hydrogen ion from the weaker acid in the secretion This tentative theory can also be seen to fit many of the facts of pancreatic secretion, where electropositive dyes are restrained, and alkali is secreted

APPENDIX I

Calculation of the distribution of phenol red between blood and gastric juice, assuming an oil phase boundary in which the dye is soluble

Phase I	Phase II	Phase III
Aqueous	Oil	Aqueous
pH 7 4		pH 1 0
Acid dye $HD \rightleftharpoons \overset{+}{H} + \overset{-}{D}$	Dye HD	Acid dye $HD \rightleftharpoons \overset{+}{H} + \overset{-}{D}$
Concentration $= C_1$	Concentration $= C_2$	Concentration $= C_3$
Dissociation $\approx \alpha_1$		Dissociation $= \alpha_3$

Distribution coefficient of dye between Phases I and II is equal to

$$(1) \qquad \frac{C_1 (1 - \alpha_1)}{C_2} = K_d$$

$$(2) \qquad C_2 = \frac{C_1 (1 - \alpha_1)}{K_d}$$

distribution of dye between Phases II and III

$$(3) \qquad \frac{C_3 (1 - \alpha_3)}{C_2} = K_d$$

Substituting C_2 from (2) above

(4)
$$\frac{C_4(1-\alpha_3)K_d}{C_1(1-\alpha_1)} = K_d$$

(5)
$$\frac{C_4}{C_1} = \frac{(1-\alpha_1)}{(1-\alpha_3)}$$

for the acid dye HD

(6)
$$\frac{\left[\overset{+}{H}\right]\left[\overset{-}{D}\right]}{[HD]} = K$$

(7)
$$\frac{\left[\overset{+}{H}\right]\alpha C_1}{(1-\alpha_1)C_1} = K$$

at pH 7 4

(8)
$$\frac{10^{-7\,4}\,\alpha_1 C_1}{(1-\alpha_1)C_1} = K \qquad \alpha_1 = \frac{K}{10^{-7\,4}+K}$$

at pH 1 0

(9)
$$\frac{10^{-1}\,\alpha_3 C_3}{(1-\alpha_3)C_3} = K \qquad \alpha_3 = \frac{K}{10^{-1}+K}$$

therefore

(10)
$$\frac{C_4}{C_1} = \frac{1-\dfrac{K}{10^{-7\,4}+K}}{1-\dfrac{K}{10^{-1}+K}} = \frac{10^{-7\,4}+10^{-4\,4}\,K}{10^{-7\,4}+K}$$

for phenol red

$$K = 1\,25 \times 10^{-8} \text{ (Clark)}$$

$$\frac{C_4}{C_1} = \frac{10^{-7\,4}+10^{-4\,4}\times 1\,25 \times 10^{-8}}{10^{-7\,4}+1\,25 \times 10^{-8}} = \frac{1\,000}{1\,314}$$

In an experimental test with this dye the predictions of the theory were veri fied   Thus it is apparent that great differences in dye concentration in aqueous phases of differing pH are not to be expected in the case of dyes with dissociation constants less than 1×10^{-8} If the distribution in such a system were the sole factor involved acid dyes should be found in gastric juice   Since they are not

it appears justifiable to conclude that some other factor plays the deciding role in allowing many acid dyes to appear in pancreatic juice while preventing all of them from coming into the gastric juice

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CORRELATION OF OXIDATION AND PHOSPHORYLATION IN HEMOLYZED BLOOD IN PRESENCE OF METHYLENE BLUE AND PYOCYANINE

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Mammalian erythrocytes though capable of glycolysis are incapable of oxidizing carbohydrates by molecular oxygen They acquire this faculty on addition of methylene blue or other reversible dyestuffs of a similar range of oxidation reduction potential (1, 2) On hemolysis both the faculty of producing lactic acid and, in presence of methylene blue, of oxidizing sugar is lost When, however, hexosemonophosphate (Robison ester) is used as a substrate instead of glucose, even hemolyzed blood cells, in the presence of methylene blue, are capable of oxidizing the substrate, as Warburg, Kubowitz, and Christian (3) have shown

Runnstrom, Lennerstrand, and Borei (4) found that in such a system consisting of hemolyzed blood, hexosephosphate ester (mono or di-) and methylene blue, addition of cozymase from yeast cells has two effects it increases oxidation and brings about a synthesis of inorganic phosphates to phosphate esters These two effects go hand in band, when respiration is suppressed by omitting methylene blue, phosphorylation does not occur either On the contrary, the amount of inorganic phosphate increases in time So, phosphate esters can in such a system either be synthesized or broken down according to whether or not respiration takes place Obviously there is an energetic coupling of oxidation and synthesis quite analogous to what Warburg designated as Pasteur reaction, namely the coupling of oxidation and synthesis of carbohydrate from lactic acid as found by Meyerhof

It has been shown already by Engelhardt (5) that a synthesis of pyrophosphate ester can take place in intact erythrocytes when their

faculty of respiration is artificially established by methylene blue   In muscle also, synthesis of a phosphate ester, namely creatin phosphate takes place under the influence of respiration   Because of the general importance of this coupling between respiration and synthesis of phosphate esters we have taken up this problem once more for the case of Warburg's system of hemolyzed blood as referred to above

This system, as stated already is able to oxidize Robison ester but no synthesis of phosphate ester takes place unless cozymase is added This holds when methylene blue is used as a catalyst   On several occasions it has been shown by Friedheim (6) that among the reversible dyestuffs of a potential range close to that of methylene blue, pyocyanine had not only a stronger but even a somewhat specific effect For this reason Warburg's system of hemolyzed blood has been investigated replacing methylene blue by pyocyanine   It will be shown that in this case respiration is coupled with phosphate ester synthesis without addition of cozymase being necessary, though addition of cozymase increases the effect even to a higher extent

The essential feature of this paper is to show that pyocyanine has an effect comparable to the one of a mixture of methylene blue and cozymase

Methods

Horse blood corpuscles were used   The serum of defibrinated blood was removed by centrifuging   After two washings with 0 9 per cent NaCl the cells were hemolyzed by addition of $1\frac{1}{2}$ volumes of distilled water and some drops of octyl alcohol were added to keep the fluid sterile   The hemolyzed cells were kept in the ice box for about 20 hours   Phosphate buffer, pH 7, was added to a final concentration M/20 to M/40 or in some experiments even lower   Under these conditions the stromata could not be removed by centrifuging (8000 R P M) Oxygen consumption was measured in Warburg's apparatus at 37°C   Methylene blue or pyocyanine was used in a concentration of 0 008 to 0 01 per cent   The pyocyanine was a synthetic preparation   It was prepared as hydrochloride and the solution carefully neutralized before use   The dyestuffs were always added to the system of hemolyzed blood and buffer before it was introduced into the respiration chamber   So the formation of methemoglobin was brought to a standstill before the beginning of the measurements   The substrate, hexosemono- or diphosphate was introduced into the side arm of the vessel and after the first reading tipped into the main compartment   After a suitable time interval the reaction was interrupted by addition of a 20 per cent trichloracetic acid   The volume of each sample was diluted with water to 100 cc   The final concentration

of trichloracetic acid amounted to 1–1 2 per cent   Estimation of phosphorus was made according to Fiske and Subbarow (7) with the modification by Theorell (8) in which a colorimeter is replaced by a step photometer of Zeiss   The amount of P is expressed in milligrams per cubic centimeter of the mixture as used in the respiration vessel

The organic acid soluble phosphates were hydrolyzed according to the method of Lebnhartz in N H₂SO₄ at 100 C   P estimations were made after boiling for 15, and for 120 minutes   Finally the total P was determined   So four different fractions of P are obtained which are designated below as I, II, III IV   The amounts of the different fractions are recorded in per cent of the total P   The fractions are

I   Inorganic P

II   Increase of inorganic P after 15 minutes hydrolysis (chiefly adenylpyrophosphate)

III   Further increase of inorganic P after 120 minutes hydrolysis (chiefly hexose diphosphate)

TABLE I

2 cc. hemolyzed cells with methylene blue or pyocyanine   At the time 0 0 2 cc hexosemonophosphate added with or without cozymase (0 02 cc)

	Methylene blue		Pyocyanin	
	—	Cozymase	—	Cozymase
Oxygen consumption 60 minutes *c mm*	182	280	155	290
Increase through the addition of cozymase *per cent*		55		87

IV   Further increase of inorganic P after complete combustion with concentrated H₂SO₄ (compounds highly resistant to hydrolysis hexosemonophosphates, etc.)

The cozymase preparation used throughout the experiments was kindly forwarded to us by Professor K Myrhack   It was purified according to the method described by him (9)   Its original strength was 330 Co units per cubic centimeter   It was kept in several sealed tubes   The activity may have somewhat decreased with aging but no current determinations of activity were made

The Action of Methylene Blue and Pyocyanine

Pyocyanine and methylene blue do not differ essentially in their effect on oxygen consumption   On addition of cozymase there is an increase of oxygen consumption with either dye (Table I)   There is, however, a distinct difference between methylene blue and pyocyanine as regards the distribution of the four P fractions   This is shown by

the record of a few experiments (Tables II and III) among the many carried out   The effect of pyocyanine alone is about the same or even somewhat stronger than that of methylene blue and cozymase, and a

TABLE II

2 cc hemolyzed cells with methylene blue or pyocyanine   At the time 0 0 15 cc hexosemonophosphate was added with or without cozymase (0 2 cc)

		Methylene blue				Pyocyanine			
		—		Cozymase		—		Cozymase	
Duration of experiment, *min*		0	180	0	180	0	180	0	180
P fractions in per cent	I	62	54 4	62 3	46 5	61 4	41 5	60 8	30 3
of P	II	2 6	3 9	0 54	6 3	0 7	6	2 3	6 75
	III	5 75	12 8	8 2	17 2	3 5	14	4	13 7
	IV	29 65	28 9	29	29	34 4	38 5	32 9	50 75

The total P was 1 1 mg per 1 cc

TABLE III

2 cc hemolyzed cells with pyocyanine   At the time 0 0 2 hexosemonophosphate was added with or without cozymase (0 2 cc)

		—			Cozymase		
Duration of experiment *hrs*		0	4	20	0	4	20
P fractions in per	I	36 7	11 2	25	35 2	4 4	26
cent of total P	II	3 7	21 6	10 9	3 1	20 2	11 3
	III	—	5 7	0 7	1 6	9 45	1 5
	IV	59 6	61 5	63 4	59 1	65 35	61 2

The total P was 0 945 mg per 1 cc

combination of pyocyanine and cozymase has a still higher effect   In the absence of a dyestuff no phosphate is bound,[1] on the contrary an increase of the inorganic phosphate may take place   It can also be

[1] The following experience may be worth communicating   As a rule, methylene blue without cozymase does not bring about any decrease of the inorganic phosphorus (Fraction I), sometimes even a small increase   In other cases there is a certain increase in Fraction I, though very small indeed   Table II represents such a case   This occurred particularly and regularly with the blood of the horse used for the experiment described in Table II   It is very likely that a substance analogous to cozymase is always present in the blood in a small concentration

seen from Table III that after a longer duration of the experiment (20 hours) a breakdown of P compounds takes place again   This is certainly due to the oxidation of the P compounds formed   However, even after 20 hours the inorganic P is far below its value at the time O

In a series of experiments the influence of the concentration of the inorganic P was tested and it was found that the absolute amount of P combined is the same whether the initial concentration of the phosphate buffer was M/40 or M/20

Table IV gives one of the experiments with hexosediphosphate as a substrate   There is a strong increase of Fraction IV during the experiment   The last three columns of the table show what happens

TABLE IV

2 cc hemolyzed cells with pyocyanine or without pyocyanine (in this case corresponding amount of distilled water was added to the hemolyzed cells)   At the time 0 0 2 cc. hexosediphosphate was added with or without cozymase (0 3 cc.)

Duration of experiments hrs.		Pyocyanine					Without pyocyanine		
					Cozymase				
		0	3	20	0	3	20	0	3	20
P fractions in per cent of total P	I	38 8	28 5	30 2	38 5	24 4	24 6	38 2	44 4	62 8
	II	20 1	20 9	10 2	24 1	17 7	12 3	22 8	21 8	12 25
	III	22 9	16 9	7 7	18 8	17 7	7 7	23 2	20 7	10 7
	IV	18 2	33 7	48 1	18 6	40 2	55 4	15 8	13 1	14 25

The total P was 1 75 mg per 1 cc.

when no dyestuff and no cozymase are present   In this case a strong increase of inorganic P (Fraction I) and no increase of Fraction IV takes place, and presence of cozymase without a dye does not change this result

Glucose alone is not attacked in the system studied   To be sure, we found a slightly higher oxygen consumption on addition of glucose

varying with the individual horse   The actual result may be best stated as follows   in absence of methylene blue an increase in inorganic P with time always takes place   in presence of methylene blue the inorganic P is approximately constant with time   sometimes there is a very small increase and sometimes a very small decrease   as though the breakdown of esters to be expected were approximately compensated by a synthesis

to the mixture of hemolyzed blood and pyocyanine and hexosemono-phosphate, also a slight increase in formation of phosphate esters was observed   But these differences were very close to the experimental errors

In some experiments it was revealed that both di- , and tetra-, methylparaphenylene diamine induce oxygen consumption in the same way as methylene blue   Phosphoglyceric acid and glycero-phosphoric acid are not oxidized by hemolyzed cells in the presence of pyocyanine, a result which is in agreement with that obtained by Runnstrom, Lennerstrand, and Borei (4) with methylene blue as dye catalyst

TABLE V

2 cc hemolyzed cells with methylene blue + 0 3 cc cozymase + 0 15 cc hexose-monophosphate with or without urethane (0 2 cc)

	————	1 5 per cent urethane	3 per cent urethane
Oxygen consumption, *c mm per 60 min*	228	214	119
Decrease, *per cent*		6	42

Some Experiments with Inhibitory Substances

Table V shows an experiment on the influence of urethane on the oxygen consumption   Even in a concentration of 1 5 per cent ure-thane, there is no effect   At 3 per cent, however, there is a drop in oxygen consumption   But even at this concentration urethane did not interfere with the synthesis of P compounds   The decrease of inorganic P during 3 hours was the same as in the control, from 60 to 44 per cent of the total P which amounted to 1 1 mg per cc

Addition of neutralized KCN in concentrations that would strongly suppress respiration in normal cells did not affect the oxygen consump-tion in hemolyzed blood + methylene blue + cozymase + hexose-monophosphate   For instance in one experiment oxygen consump-tion was 234 c mm in 90 minutes in the control and 228 on addition of N/135 KCN   In either case the content of inorganic P was de-creased after 3 hours from 64 to about 54 per cent (total P 1 1 mg per cc)

The effect of iodoacetic acid is quite different according to the par-

ticular nature of the system   In the system hemolyzed blood $+$ hexosemonophosphate $+$ methylene blue, the oxygen consumption is scarcely decreased by iodoacetate   In a system containing cozymase in addition, the increase of oxidation which should have occurred due to the cozymase, does not take place in the presence of iodoacetate   The phosphate synthesis also, which should occur on addition of cozymase, is annihilated by iodoacetate   In the system hemolyzed blood $+$ hexosemonophosphate $+$ pyocyanine, iodoacetate decreases respiration and entirely abolishes ester synthesis   The same holds true with hexosediphosphate as a substrate (Tables VII and VIII)

Sodium sulfite (neutralized) at concentrations of 0 15 to 0 3 M decreases, in the pyocyanine system, oxygen consumption by 15–20 per cent and also decreases the synthesis of esters   The effect is much less pronounced than with iodoacetate

$CuSO_4$, at a concentration of 9×10^{-5} to $1 8 \times 10^{-4}$ M, was tested in the methylene blue system   It does not decrease the oxygen uptake in absence of cozymase, but, in the presence of cozymase, decreases it to 25–30 per cent and prevents entirely the synthesis of phosphate esters

<div align="center">DISCUSSION</div>

(A) *Concerning the Effect of Pyocyanine* —The main result of this investigation is the demonstration of the fact that pyocyanine not only acts as a catalyst for the oxygen uptake of the system   hemolyzed cells $+$ hexosephosphate, but also brings about the synthesis of phosphate compounds in the system   This latter effect, to be sure, is distinctly increased by addition of cozymase, but is clearly noticeable without   No satisfactory explanation for the specific effect of pyocyanine can be offered as yet   It is, however, very suggestive to correlate it with its chemical behavior on reduction   Whereas methylene blue and similar dyes can be reduced only by a bivalent reduction, pyocyanine can be reduced by a monovalent or by a bivalent reduction   Though originally this two step oxidation of pyocyanine had been demonstrated by Friedheim and Michaelis (10) only in strongly acid solution, its occurrence in physiological pH range could be later demonstrated by Michaelis, Hill, and Schubert (11)   Here also, both steps of reduction are formed, but a considerable overlapping takes place

Though no clear insight into the causal connection between the two-step reduction and the particular nature of the catalytic effect can be ascertained as yet, this property of pyocyanine can be no accidental

TABLE VI

2 cc hemolyzed cells with methylene blue with or without iodoacetate   At the time 0 0 2 cc hexosemonophosphate added with or without cozymase (0 3 cc)

				Cozymase		
Iodoacetate	0	0 01 N	0 02 N	0	0 01 N	0 02 N
Oxygen consumption, c mm per 60 min	154	140	146	228	151	146 5

TABLE VII

2 cc hemolyzed cells with pyocyanine with or without iodoacetate (0 02 N) At the time 0 0 2 cc hexosemonophosphate added with or without cozymase (0 3 cc)

		Cozymase		Cozymase
			Iodoacetate	
Oxygen consumption, c mm per 60 min	135	193	65	65
Decrease, per cent			52	66 4

TABLE VIII

2 cc hemolyzed cells with pyocyanine with or without iodoacetate   At the time 0 0 25 cc hexosemonophosphate was added with or without cozymase (0 3 cc)

				Iodoacetate	
		Cozymase	Cozymase		Cozymase
Duration of experiment, min		0	180	180	180
P fractions in per cent of total P	I	74 3	60 5	74 8	74 1
	II	0	6 7	0 5	2 5
	III	3 7	5 8	2 8	7 6
	IV	22	27	21 9	15 8

Total P was 1 77 mg per 1 cc

feature unrelated to its physiological effect   For not only certain dyestuffs closely related to pyocyanine, especially chlororaphine (12-13), and hallachrom (Friedheim (14)), but also the dyestuffs desig-

nated as "yellow respiration ferment," (O Warburg) and flavines or lyochromes (Ellinger, and R Kuhn) show the property of two step reduction, as recently has been shown by R Kuhn and Wagner-Jauregg (15), and by Barron and Hastings (16)

It is worth while emphasizing that KCN does not affect the influence of pyocyanine either on respiration or on phosphate synthesis

(B) *Concerning the Effect of Iodoacetic Acid*—The point of attack for iodoacetic acid can be safely regarded as primarily the sulfhydryl groups, as has been suggested by Quastel (17), and proven by Dickens (18), and Michaelis and Schubert (19) These latter authors, however, pointed out that according to concentration, pH, and other more chemical specificities, amino groups also are attacked by iodoacetic acid With the possibility of these two modes of action, the effect of iodoacetic acid may be different according to circumstances, and such a consideration may account for discrepancies reported about the effect of the acid Lundsgaard (20) claims that iodoacetic acid elimi nates only the formation of lactic acid but has no influence on respira tion Nilsson, Zeile, and von Euler (21) claim that it decreases res piration also Ehrenfest (22) assumed that respiration in the presence of iodoacetic acid takes place only at the expense of split products of carbohydrates already present As the mode of action of iodoacetic acid may depend on concentration and other conditions, we wish to emphasize that the effect of the acid in our experiments should not be taken as the specific action of this acid in general, but holds for the concentration and other conditions prevailing in our experiment The following effects were observed

1 The respiration induced by methylene blue was not diminished by iodoacetate

2 In a system containing methylene blue in addition to cozymase, iodoacetate had two effects it cuts down just the increase of respira tion otherwise brought about by cozymase, and it prevents the syn thesis of phosphate esters otherwise brought about by cozymase

The effect of iodoacetate in presence of pyocyanine was twofold also (1) it decreases respiration, (2) it abolishes synthesis of phos phate esters To appreciate the first of these two effects, it should be recalled that the respiration induced by pyocyanine (without cozy mase) is not greater than the one induced by methylene blue (without

cozymase) Yet the first is decreased by iodoacetate, the latter is not But, at any event, phosphate synthesis is always annihilated by iodoacetate

(C) *Concerning the Phosphor-Ester Synthesis* —The above data can only serve as a first rough orientation concerning the chemical changes involved in the processes leading to the increase of chemically bound phosphates In the system hemolyzed cells + hexosemonophosphate + cozymase + Mb, or hemolyzed cells + buffer + hexosemonophosphate + pyocyanine, an increase of Fraction II is always found From this it may be inferred that adenylpyrophosphate is built up But also the *Fraction III increases, indicating perhaps an increase of* hexosediphosphates In the system hemolyzed cells + hexosediphosphate + Mb or + pyocyanine (Table IV), there is a strong increase of Fraction IV, formed by compounds resistant to the hydrolysis in N sulfuric acid It is difficult to understand this without assuming the breakdown of the hexosediphosphate to three- carbon compounds each carrying two phosphoric acid radicals (*cf* glycerinediphosphoric acid found in blood by Greenwald (23) and by Jost (24)) In the system hemolyzed cells + hexosemonophosphate + cozymase + pyocyanine there is also an increase of Fraction IV A more detailed discussion of the chemistry of the changes studied is, however, premature in view of the experimental material as yet available

SUMMARY

1 The system hemolyzed blood + glucose never exhibits glycolysis or, in the air, oxidation of glucose When glucose is replaced by hexosephosphate ester, addition of methylene blue causes oxidation in air

2 When cozymase is added also, the oxidation is increased, and a synthesis of hexosephosphate esters takes place

3 When pyocyanine is used instead of methylene blue, the rate of oxidation is the same as with methylene blue, but a synthesis of phosphate esters takes place without addition of cozymase

4 There is never a phosphate ester synthesis without oxidation going on, but oxidation does not necessarily go hand in hand with phosphate synthesis

5 In order to couple the oxidation process with phosphate synthesis,

two methods are available either to start oxidation by methylene blue and to add coenzyme from yeast cells, or to start oxidation by pyocyanine, in which case coenzyme is unnecessary, though it improves the effect

6 Iodoacetate always suppresses synthesis, but only under certain conditions decreases oxidation Cyanide has no effect upon either process

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THE GEOTROPIC RESPONSE IN ASTERINA

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I

With various animals differing markedly in structure it has been found that the extent of geotropic orientation upon a sloping surface is a function of the inclination of the surface   Empirically, the connection between angle of oriented progression on the surface (θ) and the tilt (α) cannot be described by the same equations in the cases of forms for which it is clear that the sensorial equivalence of impressed muscle tensions on the two sides of the body cannot be achieved in the same ways (Crozier and Pincus, 1926, 1926–27, Crozier and Stier, 1928–29, Kropp and Crozier, 1928–29, Crozier, 1929), whereas for forms dynamically similar in this respect the same formulations do apply (cf Wolf, 1926–27, Crozier and Oxnard, 1927–28, Crozier and Stier, 1927–28, Crozier, 1934–35)

It is of interest to examine from this standpoint the geotropic reactions of certain echinoderms   According to Jager (1932) *Asterias rubens* creeps geotropically upward in such a way that θ is a function of α, although not in approximate proportion to log sin α as had been found for certain other forms (Crozier and Pincus, 1926–27, Wolf, 1926–27, Crozier and Stier, 1927–28)   At first sight it is perhaps difficult to see why, in the case of a starfish, there should be any definite relationship between θ and α, in any case, it could not be expected to be of the general form obtaining in rats, caterpillars, slugs, and snails unless the gravitational pull upon the stomach (cf Wolf, 1925, Parker, 1922) could be suitably involved, deforming pressures upon the body wall would obviously seem to be ruled out in this case, although clearly indicated for holothurians (Stier, 1933, Yamanouchi, 1929)

II

Experiments were made with *Asterina gibbosa* of 1 0 to 2 0 cm radius According to Kalmus (1929) *Asterina* is negatively geotropic This is complicated, however, by spontaneous reversals to geopositive creeping which may persist for some time At any time it is possible to induce reversal of the predominant mode of response by mechanical stimulation, as by handling, removing briefly from the water, or by stirring of the water Corresponding reversal to geonegative creeping can be brought about in a similar way during a "spontaneous" phase of positive geotropism Such reversals due to handling are temporary
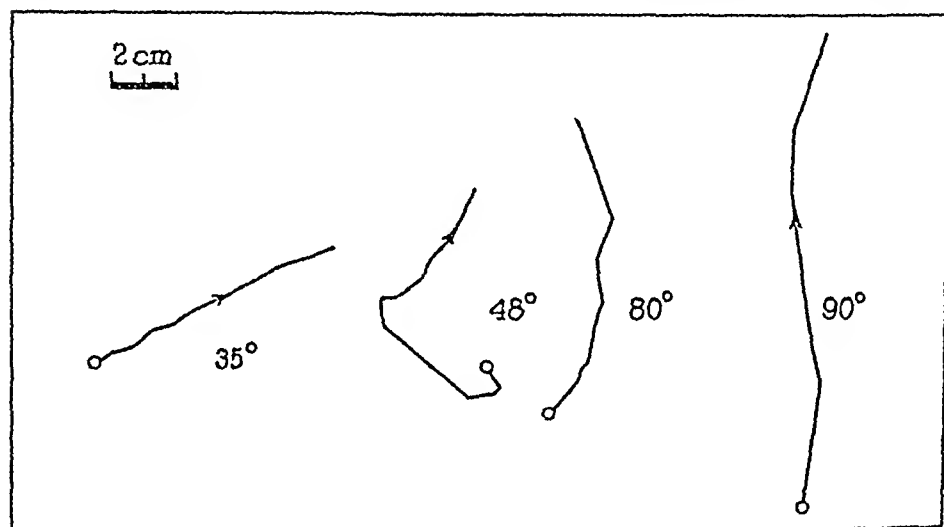
FIG 1 Specimen trails of *Asterina* upon surfaces of different slopes ($\alpha = 35°$, 48°, 80°, 90°), illustrating points discussed in the text

A sea-star was placed upon a glass plate subsequently tilted under water to a desired slope The under surface of the plate was ruled with a diamond point into a coordinate grid The path followed by an *Asterina* could then be copied accurately upon ruled paper, and the angles made by the path measured Observations were made in weak diffuse light Kalmus (1929) says that young *Asterina* are positively phototropic, "adults" negative The individuals used in these experiments were found not to be phototropic at all, at least after 24 hours in the laboratory, upon horizontal or vertical surfaces the direction of movement was not influenced by light The temperature varied between 17 8° and 20 5° Typical trails are given in Fig 1

Detailed measurements were made with six individuals   At two slopes of surface creeping was studied on the *under* side of the plate

In general character the trails secured are similar to those obtained with other geotropic animals (*cf* Crozier and Pincus, 1926–27, Crozier and Stier, 1927–28, 1928–29)   Upward (or downward) turning of the path upon an inclined plane is continued until a rather definite angle of progression is attained, in some cases the turning, due in this instance perhaps to the phenomena of "persistence" or of "continued rotation of direction" seen in sea stars, may be continued so far that, as with other forms, the path lies in a sector on the plane within which the path is not specifically oriented   This can be tested by turning the creeping plate through 90°, and observing the subsequent course of orientation   There is usually a definite elongation of the body in the direction of movement   There is no detectable tendency for the tip of one ray to be in advance, usually an inter radius lies in the direction of steady creeping   There is no evidence that the region of the madrepore tends to be ahead

Measurements of orientation angles are collected in Table I θ increases with increase of α At low slopes, the scatter of θ is greater, 10 or more paths were measured for each average   When the speed of movement is higher, at fixed α, θ tends to be somewhat greater

III

The relationship between θ and α is roughly of the type encountered in tests with a number of other forms, in that the curve is convex upward   But $\Delta\theta/\Delta \log \sin \alpha$ and $\Delta\theta/\Delta \sin \alpha$ each increase as α is made greater, $\sin \theta$ *vs* $1/\sin \alpha$ is concave   None of the equations empirically justified with other forms fits the orientation data from *Asterina*   This is of interest, since the basis for a theory of the dependence of θ upon α would also appear to be quite different, and grows out of the following considerations

The speed of progression of *Asterina* is variable, but under comparable circumstances it is also a function of the slope of the surface, increasing at higher slopes   On a horizontal surface, at 20°C , creeping was at the rate of 1 cm in 40 0 $\pm$ 4 1 seconds, upon a vertical surface, 1 cm in 23 7 $\pm$ 5 5 seconds

Kalmus (1929) made one experiment in which a cork float was attached by a thread to the dorsal side of *Asterina*   He found that in

consequence of the upward pull, the sea-star moved downward upon a vertical surface, in agreement with the effect upon a turntable This we have repeated   A fragment of cork is attached at one end of a thread 2 or 3 inches long, the other end sewn through the dorsum of an *Asterina*   The volume of cork is adjusted to give about the maximum pull supportable, under water, by a few of the tube feet   On a surface

TABLE I

Mean orientation angles (θ) for *Asterina gibbosa* as a function of slope of surface (angle α)

α	θ
degrees	*degrees*
30	32 5 ± 1 6
	24 7 ± 4 9
35	28 3 ± 2 5
40	42 8 ± 3 6
	38 8 ± 4 1
	27 0 ± 2 7
	36 2 ± 2 2
45*	45 0 ± 2 5
46	37 3 ± 4 4
48	47 1 ± 1 4
50	42 8 ± 4 1
57	53 0 ± 2 5
60	57 8 ± 2 3
63	57 5 ± 4 0
70	60 0 ± 2 3
73	75 3 ± 2 1
75*	70 0 ± 1 5
80	72 9 ± 2 1
85	70 0 ± 2 5
90	87 0 ± 1 8
	80 6 ± 1 4

* Creeping on *under* side of glass plate

inclined at $\alpha = 70°$, the animal goes slowly downward, at $\theta = 90°$, this is also true at $\alpha = 30°$ or lower   Repeated reversals of the inclination of the plate are followed by prompt and precise downward movement   When the upward pull is stopped, by holding the cork, movement continues downward for about 1 minute or so, but much more rapidly, then the animal orients upward through an arc, and at

an appropriate θ, if the upward pull is restored, downward movement is immediate   If the pull is made to act horizontally, by passing the thread over a rod, the *Asterina* creeps horizontally toward the other side   In these respects the direction of movement is controlled precisely as in the case of a gasteropod (Crozier and Navez, 1930)   In relation to the question of the connection between θ and α the demonstration can be made more illuminating by using smaller cork floats, giving a lesser upward pull   Upon surfaces of intermediate slope (e g , 55°) the *Asterina* then creeps downward, but at $\theta < 90°$, if the pull of the float be removed, it orients upward, but at a higher θ than on the downward path   It was pointed out before that in downward and in upward progression on the sloping surface (without the attached cork) θ is the same   The effect of the attached float is the same when the sea star is creeping upon the under surface of the plate, in this case the direction of pull is in the plane of the surface

The direction and the magnitude of the tension exerted upon the tube feet thus appear to be unquestionably responsible for the direction and for the limitation of the extent of gravitationally induced orientation under these conditions   The "spontaneous" reversals of orientation, and the reversal to positive geotropism seen after mechanical disturbance, present a problem of a different order, presumably there must be involved a central nervous reversal of sensory effects similar to that concerned in the reversal of galvanotropism (Crozier, 1930) in sea stars and other echinoderms

IV

In view of these facts it is necessary to consider that upward orientation involves movement in the direction away from a lateral pull   In Fig 2 the gravitational component acting laterally to the *Asterina*'s path is labelled N, that in the line of movement M We have to suppose that when the fraction of the total pull parallel to the surface ($W \sin \alpha$) which corresponds to M reaches a certain value as result of orientation, the laterally acting component (N) is without further influence, this merely corresponds to the fact that there is a threshold slope of surface for orientation   It is further to be supposed that when α is varied the limiting fraction ($M/W \sin \alpha$) will be a function of $W \sin \alpha$, and presumably directly proportional to it, since the

orientation angles are the same for *Asterina* of different sizes, W, the weight of the animal, may be ignored   It cannot be supposed that component N has merely to be reduced to a certain minimum or threshold value, N is measured by $N = \sin \alpha \cos \theta$, and is neither constant at orientation nor a simple function of $\sin \alpha$[1]   We then write $\Delta (M/\sin \alpha)/\Delta \sin \alpha = const$ as giving a reasonable statement of expectation concerning the geotropic performance of *Asterina*   Since (Fig 2) $M/\sin \alpha = \sin \theta$, we have $\dfrac{\Delta \sin \theta}{\Delta \sin \alpha} = const$ as the formulation
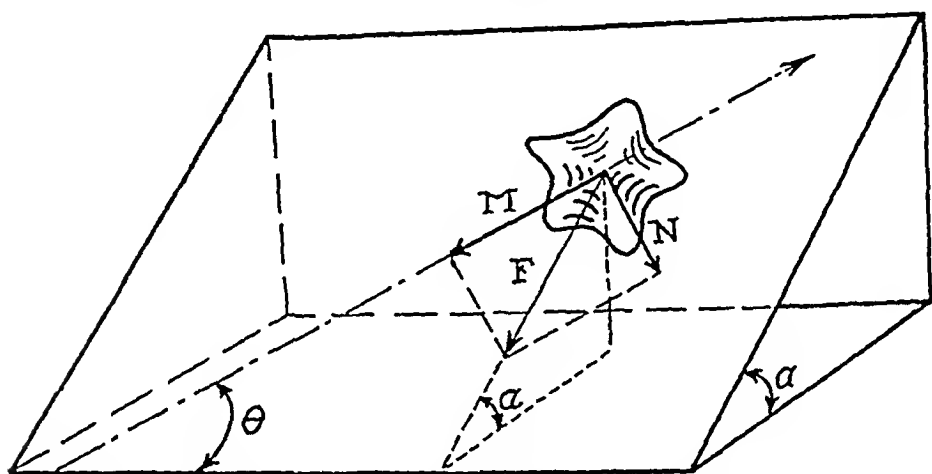
Fig 2 The effective gravitational pull F upon an *Asterina* on a surface inclined at angle α is resolvable into two components M, in the line of progression at angle θ, and N acting laterally to bring about further orientation until, as discussed in the text, M/F acquires a magnitude proportional to F

it is expected should be followed   Fig 3 shows that the equation is very nicely obeyed   At low slopes θ is in small samples more variable (*cf* Table I) than at high slopes, as is the general rule (Crozier and Pincus, 1926–27, Crozier, 1934–35, etc), and the points in Fig 3 consequently spread less and less widely as $\sin \alpha$ increases

In Jager's data (1932) for *Asterias rubens* the same rule applies

---

[1] *Cf* Crozier, 1934–35   von Buddenbrock (1931) and Jager (1932) took $\sin \alpha \cos \theta$ as the sine of the angle of tilt or of the transverse axis of the oriented animal   The former argued that this latter angle tended to be made constant in geotropic progression, which is not correct   Neither noticed its significance as a force vector

accurately if one supposes, as I believe one must, that at $\alpha = 30°$ the group of θ s above $65°$ represents paths in the zone (*vide supra*) of no orientation (*cf* Jager's Fig 22) This is shown in Fig 4
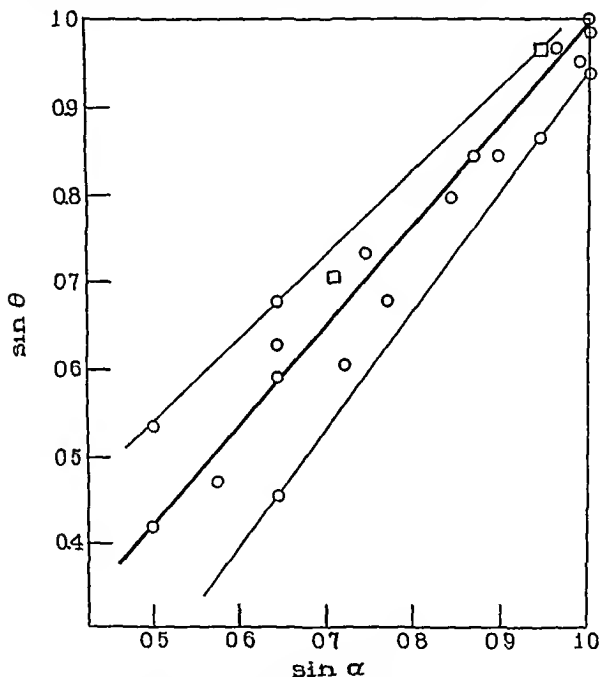
FIG 3 Showing that $\Delta \sin \theta / \Delta \sin \alpha$ is constant for *Asterina* The scatter of mean θ s is progressively greater at lower values of $\sin \alpha$ Data in Table I

This interpretation might be tested if the animal could be converted into one with bilateral symmetry Mangold (1909) noted that single arms of *Asterina* creep upward upon an inclined surface By cutting off four of the five rays and a strip of the disc connecting

them, one has a preparation in which most of the remaining tube feet
are upon the intact ray   After a day such a preparation creeps well,
all the tube feet functioning actively   It usually creeps with the tip
of the ray foremost, and in the line of the axis of the ray   This is
most often observed, however, if it is upon a steeply inclined surface,
and is indeed then the predominant condition   After effects of han-
dling have disappeared, it orients upward from an initially horizontal
path, and creeps upward with the tip of the ray in advance, the course
traced by the oral opening is in line with the axis of the ray, except
for brief intervals   If the plate be reversed, orientation is reversed
promptly   Orientation usually involves actual bending of the ray
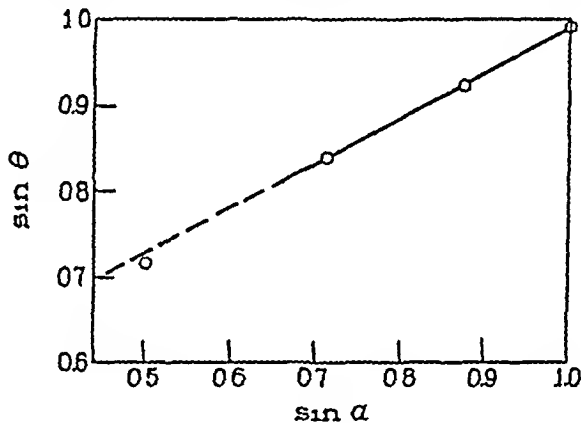
Fig 4   A similar relationship may hold for the geotropic orientation of *Asterias*,
based on the data of Jager (1932), the measurements at $\alpha = 30°$ being corrected
as explained in the text

Occasionally the path swerves to a steeper θ, and the rotation continues
until the corresponding θ on the other side of the vertical mid-line is
reached   In positively geotropic creeping following handling the
orientation of the ray is sometimes lost, but tends to be regained, the
loss of director action by the ray appears when the path is at about
$\theta = 90°$ and the animal is creeping rapidly   This is the mode of orien-
tation to be expected if the axis of progression is definitely polarized
and if the pull upon the tube feet is to be adjusted in the way here
assumed by the attainment of a path at angle θ which is a function of
the slope of the surface   The θ's observed with the single-ray prepara-
tion were generally lower than with the intact *Asterina*, this may be
due to a lowering of the threshold for response to tension

SUMMARY

Upon a surface inclined at angle α *Asterina gibbosa* orients upward during negatively geotropic creeping until the average angle (θ) of the path is such that $\Delta \sin \theta / \Delta \sin \alpha = const$ This is true also in positively geotropic movement The direction of orientation may be temporarily reversed by mechanical disturbance The variation of θ is greater at low slopes Tests with directed impressed pulls, due to an attached cork float, show that the pull upon the tube feet is of primary consequence for the determination of θ When the component of gravitational pull in the direction of movement reaches a fraction of the total pull which is proportional to the gravitational vector parallel to the surface, the laterally acting component is ineffective On this basis, it follows that $\Delta \sin \theta / \Delta \sin \alpha = const$

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ON REVERSAL OF GEOTROPISM IN ASTERINA

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I

The normally negative geotropism of *Asterina gibbosa* (Loeb, 1900, Mangold, 1909, Kalmus, 1929, Crozier, 1934–35) can be temporarily reversed to positive by mechanical stimulation due to handling, or stirring of the water (Crozier, 1934–35)   The negative geotropism is unquestionably a response to tension upon the tube feet (Crozier, 1934–35), the sea star can be forced to creep persistently downward by attaching to its dorsum a cork float, and the relation between angle of oriented movement and slope of surface is quantitatively described on the basis of this view   It was suggested that the reversal of geo tropism following mechanical disturbance might be understood as an instance comparable to those in which "reversal of inhibition" brings about inversion of galvanotropism in echinoderms (Crozier, 1930) and certain other forms (Crozier, 1926–27)

This interpretation is tested by observing the geotropic performance of *Asterina* under the influence of strychnine, with and without cork floats attached   This particular test is necessary because in absence of a result with the added tension due to the float it could not be told whether one had to do merely with an effect of the strychnine in simply making the animal more sensitive to mechanical (tension) excitation

In considering the effect of a drug (or other altered condition) upon an element of behavior it is necessary to keep it in mind that the influence of the reagent has a characteristic time course   It is only the question of reversal of geotropism which is of interest here, not the mode of action of the drug, but the time course varies with the concentration of strychnine and with the size of the *Asterina*   The inter

val of developing strychninization within which it is possible to look for the evidence required is rather sharply limited   On the one hand, sufficient development of the effect of strychnine must be attained, on the other, the tests must be made before strychninization has advanced so far that the tube feet no longer attach to a substratum

Sea-stars placed in dilute solutions of strychnine sulfate characteristically show dorsal flexure of the rays, with failure of attachment of the tube feet (Moore, 1918–19, 1919–20, Crozier, 1930), particularly when stimulated   This represents a reversal of the typical response The galvanotropic orientation of the rays is with the oral surface toward the cathode, the rays being extended toward the cathode   The tube feet extend toward the anode, and creeping is in this direction Under the influence of strychnine all this is quite reversed (Crozier, 1930)   The effect of pilocarpine is similar, but not so pronounced The reversal of galvanotropic response is accompanied by reversal of reaction to mechanical stimulation   Similar results hold for other forms (Crozier, 1926–27, Fries, 1927–28, Clarke and Wolf, 1932–33), and have been interpreted in terms of reversal of inhibition under strychnine

II

Asterina in dilute strychnine sulfate reach a state in which the tube feet release attachment as result of application of even a slight touch or pressure, the rays and disc curve dorsally   Before this stage the tube feet may be caused to attach to a glass plate, although very slowly   They are then found to be positively geotropic, although progression is slow   Small wire hooks caught on the dorsum produce no change in the direction of creeping, despite the added weight There is no reversal to negative geotropism as result of handling   If slightly more deeply under the influence of strychnine, such reversal to upward movement (temporarily) may be obtained by stirring the water with a glass rod   In some cases upward and downward movements alternate "spontaneously" for a short while   When emerging from strychninization, there is a period in which the now once more geonegative *Asterina* is very easily caused to become positively geotropic by agitation of the water

The reversal to positive geotropism under the action of strychnine might be regarded as simply the result of "increasing irritability" But a small cork float may be attached to the hook in an *Asterina s* disc while creeping geopositively after being exposed to strychnine The size of the cork is adjusted to give a moderate upward pull despite the weight of the hook Without strychnine, or in an inappropriate phase of the action of the drug, the pull of the float leads to downward creeping (Crozier, 1934–35) It now results, however, in *upward* movement Depressing the cork, or cutting the thread which holds it, leads to downward movement once more

III

There is thus a stage in the strychninization of *Asterina* during which the usual negative geotropism is reversed The effect of an upward pull is also reversed This accompanies reversal of response to touch, and must be regarded as involving reversal of inhibition The effect of tension on the field of tube feet is normally to produce an orientation against the pull, no matter bow strong it may be up to the point where the tube feet can no longer maintain attachment Under strychnine this response is reversed Consequently the reversal cannot be regarded as due merely to an intensification of the sensory effect of tension, but must be interpreted as due to a true reversal of inhibition in the coordination of effector movements, comparable to reversal of galvanotropism (Crozier, 1926–27, 1930) The temporary reversal of geotropism normally resulting after mechanical stimulation, which is a phenomenon seen in other forms also (e g , Fraenkel, 1929), must be understood in the same way

SUMMARY

A certain level of strychninization induces in *Asterina* reversal of geotropism from the normally geonegative movement to a persistent downward creeping The effect of an attached float producing upward pull is to induce upward creeping, under these conditions, whereas normally it leads to downward movement This reversal cannot be regarded as due to a mere intensification of the sensory effect of tension It must be understood as representing a true re-

versal of inhibition   The temporary reversal of geotropism following mechanical disturbances (in the absence of strychnine) is interpreted in the same way.

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ORIENTATION BY OPPOSED BEAMS OF LIGHT

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I

From the elementary postulate that in phototropic orientation the turning movements cease when excitation is made sensibly equivalent upon the two sides of a symmetrical organism, equations have been derived (Crozier, 1925–28, Mitchell and Crozier, 1927–28) which describe properties of the oriented position under the influence of two sources of light   With a moving organism the situation is most susceptible to useful analysis when beams of parallel rays are con cerned (Mitchell and Crozier, 1927–28), since then an oriented position is assumed in definite relationship to the axes of the beams   With beams opposed at 180°,

$$\tan \theta = (\cot H/2) \frac{(I_1 - I_2)}{(I_1 + I_2)} \tag{1}$$

where intensity $I_1 > I_2$ and, for a negatively phototropic animal, θ is the angle of orientation away from I_1, the angle H, loosely termed a "head angle," is the average effective angle between the bilaterally disposed photoreceptive surfaces (Crozier, 1925–28)   The magni tude of H must be expected to change if the amplitude of side to side movements of the anterior end of the body, or if the random or periodic frequency of such movements, depends upon the illumination or other conditions (e g, temperature, or concurrent geotropic excita tion), it need not correspond to any obvious feature of the structure of the organism (Crozier, 1925–28)

It was pointed out that certain interesting comparisons might be made by contrasting values of H obtained from measurements of θ with two beams of light directly opposed with similar data secured when the beams cross more acutely, say at 90°   For the latter case,

$$\tan \theta = \frac{I_2 \tan H + I_1}{I_1 \tan H + I_2} \tag{2}$$

(*cf* Mitchell and Crozier, 1927–28)   If H were strictly constant, it should appear nearly identical for the two cases (unless complicated by effects of refraction)

Experiments were made with larval blow flies of two species, *Calliphora erythrocephala*, 4 days after emergence and 7 mm to 9 mm in

TABLE I

Mean angles of orientation for larvae of *Calliphora* under the influence of two beams of light crossing at 90°   Two series of measurements, six larvae used in each series, six observations on each larva at each intensity

I_1	I_2	θ		H
		Mean	P E	
millilamberts	*millilamberts*			
3781	3560	50 86° ±	0 31°	57 5°
	3260	59 56	0 14	58 3
	3008	64 98	0 15	55 3
	2752	69 47	0 08	51 9
	2207	80 33	0 25	49 3
	1910	86 16	0 13	50 5
	1801	88 85	0 13	49 5
	1621	89 22	0 13	45 3
3897	3599	51 22° ±	0 93°	49 4°
	3210	58 74	1 4	46 7
	2995	65 06	1 3	50 4
	2599	72 21	1 2	47 5
	2151	79 81	1 1	44 9
	1898	86 92	0 45	47 9
	1755	87 95	0 32	45 7
	1609	89 03	0 20	43 4

length, and a species of *Lucillia* of the same age but 6 mm to 8 mm in length   The first was used by Patten (1914), for larvae of the size he employed, $H = 85°$ under the particular conditions of excitation (Crozier, 1925–28)   We used smaller larvae, our chief point being to compare values of H with lights opposed and crossing at 90°, and to compare values of H for two morphologically different larvae   Larvae of *Lucillia* are more sharply pointed at the anterior end   We ex-

amined the orientation both with beams opposed at 180° and crossing at 90° The computed values of H need not be expected to be the same for the two situations, since if the photoreceptors are located beneath the surface of the larva the refraction of incident light will differ significantly It was expected that variation in H might be correlated with intensity of illumination, or with θ
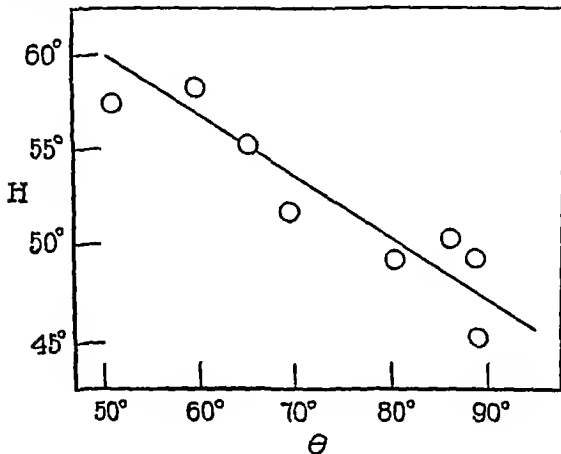
Fig 1

From two similar electric bulbs, within housings, light was allowed to fall upon a square platform on which the larvae crept Trails were recorded by placing a drop of methylene blue at the posterior end of the maggot The lights were mounted upon tracks of an optical bench, in a series of measurements one lamp was moved to different distances from the observation platform The sources of light were slightly above the level of this platform, to obviate shading of the larva's anterior end at higher angles of orientation At the position occupied by a larva the illuminations from the two sources were measured with a Macbeth illuminometer The shape of the

larva changes during its development, in a way which presumably affects H, consequently no exact agreement could be expected between successive series of determinations   The values of θ were read from the trails, to the nearest half degree

TABLE II

Orientation angles for *Calliphora* larvae of size corresponding to those used in Table I, but with beams of light directly opposed, five larvae, six readings on each

I_1	I_2	θ		H
		Mean	P E	
millilamberts	millilamberts			
3972	3765	3 45° ±	0 31°	60 9°
	3107	8 11	0 65	81 4
	2777	12 00	0 73	80 1
	2474	17 35	0 73	73 6
	2001	26 21	0 85	67 7
	1763	35 42	0 56	57 0
	1407	38 51	1 1	62 1
	1100	43 60	0 67	60 8

TABLE III

Orientation angles for *Lucilia* larvae, with beams of light crossing at 90°, five larvae, six readings on each

I_1	I_2	θ		H
		Mean	P E	
millilamberts	millilamberts			
3019	2960	45 7° ±	0 52°	21 2°
	2443	53 6	0 73	20 7
	2279	56 4	0 65	20 7
	2098	59 7	0 54	21 1
	1580	69 3	0 64	20 6
	1076	78 8	1 06	18 1
	718	86 4	0 53	20 1
	602	87 8	0 39	18 1
	555	88 4	0 30	17 8

II

With *Calliplora* larvae two series of measurements were made (Table I) with light beams crossing at 90°   In each series there is a

definite decline of H as θ increases (Fig 1), that is, as the total illumination $(I_1 + I_2)$ declines   This may be due to the fact that chance movements, especially toward the less intense light, are more frequent This does not appear in the variability of the orientation angle θ, since the error in θ is perhaps chiefly in the mechanics of its measurement, with a wider range of intensities of illumination (cf Crozier, 1925–28), $P\,E_{\theta}$ and H might each be expected to go through a maximum   In the second series, with slightly younger larvae, H is consistently smaller than in the first set, and $P\,E_{\theta}$ larger   At high magnitudes of θ, however, H tends to agree

A third series of observations employed lights directly opposed (Table II)   Here again H declines as θ increases, the values are consistently above those in Table I (cf Fig 1)

The anterior end of the larva of *Lucilia* is much more sharply pointed than is that of *Calliphora* larvae of the ages used   This need not be taken to imply that the effective inclination of the photoreceptive surfaces (of the imaginal discs?) is correspondingly acute, and no measurements were made of the character of the side to side movements of the anterior end in creeping, which could affect the computed magnitudes of H Tests were made of orientation with beams of light crossing at 90°   The results are contained in Table III H again declines as θ increases (or as $[I_1 + I_2]$ decreases (Fig 1))   With the more sharply pointed *Lucilia* larvae the magnitudes of H are less than half as great as with *Calliphora*, it does not seem probable that this contrast can be due entirely to differences in frequency or extent of random movements of the anterior end, it must rather be ascribed to a real difference in the angular inclination of the receptive surfaces in the larvae of the two species

III

SUMMARY

Computations of the effective angular inclination (H) of the photoreceptive surfaces of the two sides, based upon measurements of orientation angles under the action of beams of light directly opposed or crossing at right angles, show that with larvae of *Calliphora* and of *Lucilia* H declines as the total illumination decreases (i e, as the

angle of orientation away from the more intense light increases) H is greater with the two lights opposed at 180°, this may be due to the difference in refraction   For the more sharply pointed larvae of *Lucillia*, H is less than half as great as in *Calliphora*

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ELECTROPHORESIS OF STEROLS

II Ergosterol

BY LAURENCE S MOYER

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Although such a large literature on the electrophoretic behavior of cell surfaces exists (1), our knowledge of the ζ potentials of many of the important constituents of these interfaces is meager   Partly for this reason, attempts to identify surface components from the electrokinetic properties of living cells have met with difficulty   These uncertainties have to a large extent been removed by the numerous investigations now available on protein systems (1, 2)   Among the lipids, however, only lecithin and cholesterol have been investigated to any extent (3)   The previous work on cholesterol has been summarized in an earlier paper (4)   Partly as a comparison and partly because of its own importance in cell activities, it was decided to investigate ergosterol from an electrokinetic standpoint

Methods

Ergosterol of a high degree of purity was secured from Dr Charles E Bills   This contained (in addition to the one molecule of water of crystallization) about 4 per cent of α-dihydroergosterol (5)   Due to the limited quantity available further purification was not attempted   The material consisted of snow white crystals and showed no signs of being contaminated with oxidation products   While in our possession it was always stored at 0°C in the dark   To prepare suspensions the method used for cholesterol (4) was employed   This consisted in grinding the sample for an hour with pure ice in an agate mortar at $-10°C$ The resultant powder was kept in the dark at $-10°C$ until needed   No changes were noted on storage at this temperature

This pulverized sterol when dissolved in chloroform exhibited the characteristic red to blue reaction of ergosterol with trichloracetic acid (6) With mercuric acetate in nitric acid the color change was from transient red to yellow This is indicative that the material was not changed or oxidized by the treatments employed to produce suspensions (7) As needed, suspensions of the powdered material were made in distilled water Acetate buffers of constant ionic strength (μ = 1/150) were used A modified Northrop-Kunitz microelectrophoresis apparatus was employed in the determination of mobilities The rest of the technique concerning buffer preparation, electrophoresis, quinhydrone pH measurement, etc, is described in previous papers (4, 8, 9) Following the suggestion of Abramson (1), temperatures were corrected by multiplying the velocities at the temperature t by the fraction η_t/η_{25} where η is the viscosity of water at t and 25°

EXPERIMENTAL

The experimental results are shown in Fig 1 It was found that ergosterol becomes isoelectric at pH 3 1 instead of pH 3 2, as previously found for cholesterol (4) The material was also more variable in its behavior As before reversal in sign was noted but not to any great extent The smooth line running through the points is the curve fitted by the use of Langmuir's adsorption equation to the data for *cholesterol* The excellent agreement between the two sets of data shows that within the limits of experimental error the two sterols are identical in electrophoretic behavior The extrapolated dash line is merely to indicate sign reversal

Several explanations might be advanced to account for this similarity (1) the presence of an ampholyte as an impurity common to both, (2) the occurrence of sufficient ergosterol as a natural contaminant of the cholesterol (3) a slight dissociation of OH^- ions from each sterol (4) preferential adsorption of H^+ or OH^- ions The first possibility seems to be excluded upon consideration of the high melting points of the cholesterol preparations and the extensive purificatory treatments which they received (4) As shown by Rosenheim and Webster (10) and others ergosterol occurs naturally in preparations of cholesterol Two factors militate against the possibility of ergosterol causing the ζ-potential of cholesterol, (1) the small quantities

reported—amounting to less than 1 part per thousand of cholesterol (10, 11) and (2) the method of preparing the suspension by crushing which gives no chance for the ergosterol to leave the crystal and coat the surface Further experiments were undertaken to test this assumption

Cholesterol from spinal cords of cattle which had been carefully purified by saponification (4) was also refluxed with norit after the procedure of Rosenheim and Webster (10) After filtration, the colorless alcoholic solution was allowed to crystallize (m p 148 5–149° corr), vacuum-dried, and made into a suspension by grinding No changes in velocity or isoelectric point were found Although Bills,
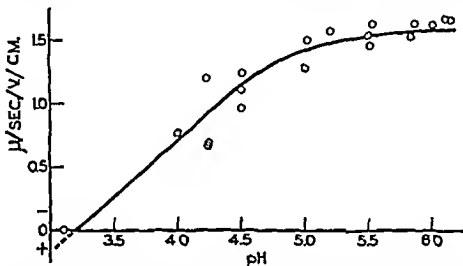
FIG 1 Electrophoretic mobility of ergosterol The circles are the experimental points for ergosterol, the smooth curve is the theoretical curve for cholesterol

Honeywell, and MacNair (11) have shown that this procedure does not remove every trace of ergosterol, a change in behavior would be expected if ergosterol were the causative agent Furthermore, the Rosenheim and Callow (7) trichloracetic acid test for ergosterol was negative with all of the author's preparations of cholesterol

Both sterols have a single OH radical The possibility of ionization as a base at this group to the extent needed to produce charge reversal seems slight Incidentally, Remesow (12) has found that particles of the cholesterol esters of stearic, oleic, and palmitic acids are negative at all pH values

The fact that the two sets of data are both fitted by the same Langmuir equation (4)

$$V = \frac{\alpha\,\beta\,[\text{OH}]}{1 + \alpha\,[\text{OH}]} \tag{1}$$

(where $\alpha = 8\,14 \times 10^9$, $\beta = 1\,57$, and V is the velocity in μ/sec /volt/ cm), which has also been employed with success by Abramson and Muller (13) and Abramson (1) in their studies of the charge of "inert" surfaces, is evidence for preferential adsorption of H^+ or OH^- ions, as the basis of the reaction

Such adsorption would tend to change σ, the effective surface charge per unit area   By the use of the Gouy and Helmholtz equations (4, 13, 14) it can be shown that if κ, the reciprocal of the effective thickness of the double layer, remains constant and $\kappa\,r \gg 1$ (where r is the radius of the particle), σ becomes directly proportional to V for small values of V Hence V can be used in equation (1) instead of σ [1]   Activities should more properly be used in this equation instead of "concentrations" but since uncertainty still exists concerning the correct value for the calomel electrode (E_0) and the liquid junction potential (E_D) involved in pH measurement (15), both the experimental points and the theoretical curve in Fig 1 are expressed in terms of the Sørensen standards proposed by Clark (16), with the assumption that $p\text{OH} = -\log_{10}C_{\text{OH}}$ If a value of E_0 which will give $p\alpha\text{H} = -\log_{10}a_{\text{H}^+}$ (so that the values of a_{H^+} will nearly equal values of the mean ionic activity of the acetic acid in the solution) is finally agreed upon, the value for β in equation (1) will remain the same and the only change necessary to replace pH by $p\alpha\text{H}$ in Fig 1 will be a slight shift of the abscissa to the left for a distance of about 0 04 pH unit   The C_{OH} is used instead of the C_H simply for convenience As stated before, the isoelectric point was not included in the calculations but the extrapolated curve ran through it

The mere fact that the Langmuir equation fits one set of data does not prove an adsorption mechanism but the goodness of fit for *both* of these different crystals seems to strengthen the evidence   It seems significant that Abramson (17) has found that the levorotary forms of the insoluble amino acids, cystine, tyrosine, and aspartic acid, all reverse their sign of charge between pH 2 3 and 2 5   The present

[1] This can only be done if the error introduced by substituting $f(I)$ for sinh $f(I)$ is negligible

communication should be of interest in the analysis of the surfaces of latex particles (8, 9, 18)

SUMMARY

The electrophoretic behavior of powdered ergosterol crystals is identical with that previously found for cholesterol within the limits of experimental error Evidence for an adsorption hypothesis is presented to explain this phenomenon

The author is indebted to Dr Charles E Bills and Prof William Seifriz for their kindness in supplying the ergosterol used in these investigations and to Prof R A Gortner for his encouragement and helpful suggestions

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ON THE RELATION OF DIRECT CURRENTS TO CONDENSER DISCHARGES AS STIMULI

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The great difficulty of determining from direct observation the nature of the structures and constituents concerned in the excitatory process in tissue compared to the relatively great ease with which the time intensity relations for electrical stimuli may be measured makes it necessary that a knowledge of the mechanism be deduced from a mathematical analysis of the latter observations

It is essential that any useful mathematical analysis of the excitatory process should predict equations for the time intensity relations for different types of stimuli which are mutually consistent, so that the constants appearing in the excitation equations for one type should also appear in the equations for another type applied to the same tissue during the same experiment   It is the present purpose to discuss the results of experiments in which the time intensity curves for both direct currents and condenser discharges were obtained in succession on the sciatic gastrocnemius preparations of the frog so that the tissue remained in approximately the same condition throughout

It was shown previously (1932 a, b, c, d, 1934) that solutions of the differential equation,

$$\frac{dp}{dt} = KV - kp \tag{1}$$

where p is the local excitatory process, V the stimulating voltage or current, and K and k are constants, very adequately represent time-intensity curves obtained by different experimenters on different tissues   For direct current stimuli the solution is,

$$\log \frac{V}{V - R} = kt + C \tag{2}$$

where C is a constant and R the rheobase   For condenser stimuli the solution is

$$\frac{V}{R} = (crk)^{\frac{1}{crk-1}}$$ (3)

where c and r are the capacity of the condenser and the resistance of the circuit respectively

These equations must represent adequately not only their proper data but it is further required as an assurance of their validity that the constant k shall be the same whether it is evaluated from direct current data or from condenser data on the same tissue at the same time   When this point was considered previously (1932 b) only one set of data by Lapicque was available and the agreement in that case was sufficiently good   The present data were obtained to test further the same type of agreement

Apparatus and Method

The direct current stimuli were obtained from an arrangement consisting essentially of a hard rubber disc attached to a heavy brass disc for rigidity   In the face of the rubber disc whose diameter is about 12 inches is set a brass wedge with its base toward the centre and its point toward the periphery of the wheel This wedge extends from close to the hub of the disc to its outer edge   Its base is about three-fourths inches in width   The wheel is set on a shaft so that it can be driven by a synchronous motor at about 800 R P M   The disc containing its brass wedge is mounted and ground in such a way that its face rotates with no greater wobble than 0 001 inch   Set on a screw carriage to enable its movement to and from the centre of the wheel is a pair of brushes of lubricated carbon, the one above and separated from the other   The brushes are carried in close fitting slots and are held against the wheel by means of coil springs which also act as electrical contacts   As the disc is turned both the brushes are in contact with the brass wedge for a duration depending on the position of the carriage   When the carriage is near the centre the wedge is wide and its linear velocity is low so that the duration of contact is long   Toward the periphery of the disc the opposite is true   The displacement of the brush carriage required to alter the duration from 0 0001 to 0 001 second is about 40 mm , from 0 001 to 0 002 second about 20 mm , from 0 to 0 0001 second about 10 mm , so that in all the scale up to 0 002 second is about 7 cm long and accurate setting is comparatively easy   The machine may be calibrated either statically, by measuring the angle through which the brushes remain on the brass and calculating the time from the speed of rotation or, by measuring the current registered on a direct current meter when the circuit is closed and the wheel rotating continuously   In the latter case the

ratio of the current when the wheel is going to that when it is stopped with the brushes on the brass gives the time of contact directly when the number of contacts per second is known

Since a high voltage, i e in excess of 100 is required to ensure negligible resistance at the brass to carbon contact 180 volts was used throughout with a series resistance of about 200,000 ohms The stimulus was derived from a variable non inductive resistance in the circuit acting as a potentiometer A similar resistance in series was used as a compensator to keep the whole resistance constant.

The machine when operating continuously gives about fifteen stimuli per second Such a spacing should not give latent addition but there may be changes of excitability following the responses For this reason a switch was used so that only one to three stimuli were given each time It was found by photographing the impulses with an oscillograph that the switch could be manipulated so as to give one or two stimuli and the machine was used in this way

The advantages of the machine are that a time-intensity curve can be obtained quite rapidly, i e in about 5 minutes, as there are no switches to set and the durations can be changed without stopping the wheel Another is that there appear to be no harmful extraneous accumulations of charge due to friction and another that the capacity of the brushes can be made negligibly small so that the circuit has very low capacity The effect of this capacity is avoided in any case by short circuiting the tissue while the brush circuit is being made Its disadvantages are that the disc must be very carefully made so that it will be smooth enough to avoid chattering of the brushes Also the currents tend to become irregular with durations shorter than about 0 0002 second The reason for this has not been fully determined so the measurements have been confined to greater durations

Condensers with mica dielectric of good quality and accurate to at least 2 per cent with the smallest capacities were used for obtaining the voltage-capacity data

In each case the circuit used for both types of stimulation was kept exactly the same except in so far as it might be altered by changes in the tissue

Silver chloride electrodes of about 0 5 mm diameter were used throughout The nerve trunk was suspended vertically in air in an enclosed chamber so that it was against the electrodes whose variable separations are given in the data The chamber was partially filled with water so that its contained air would be kept nearly saturated with water vapor The responses of the muscles were used to determine the adequacy of the stimuli.

The resistance of the tissue, which is required for Equation 3, was measured in each case by determining the rheobase with the tissue alone then with 50 000 ohms in series, then with 75 000 ohms in series and then with 115 000 ohms in series These rheobases when plotted against the resistances in series yield a straight line whose intercept gives the resistance of the tissue. The accuracy of the method apart from considerations of polarization depends on the constancy of the rheobase during the time about 2 minutes required to make the readings The durations of the rheobasic currents are probably about 0 002 second so that the polarization should not be important.

In Table I are given the results of eighteen experiments in which the k values of Equations 1 and 2 were obtained from complete time-intensity and capacity-intensity curves taken in succession as rapidly as possible   Apart from the natural differences of the different preparations and of the room temperature which varied from day to day, the experiments differ only in the separation of the electrodes   Only those experiments are reported in which a given measurement, such

<div style="text-align:center">TABLE I</div>

Experiment	Separation	D C. k	Cond k	Cond $k/$D C k
	mm			
(*a*)	32	1633	1625	1 00
(*b*)	25	2300	2290	1 00
(*c*)	20	2070	2050	0 99
(*d*)	20	1805	1975	1 09
(*e*)	20	1886	1945	1 04
(*f*)	20	3130	3260	1 04
(*g*)	20	2010	2095	1 04
(*h*)	15	1783	1755	0 98
(*i*)	15	2588	2500	0 96
(*j*)	14	1842	2790	
(*k*)	11	2830	2945	1 04
(*l*)	11	2935	3750	
(*m*)	5	3565	3590	1 01
(*n*)	5	2645	4260	
(*o*)	5	2530	4195	
(*p*)	4 5	2530	2750	1 08
(*q*)	4	3160	2990	0 91
(*r*)	2 5	1585	1620	1 02
Average				1 015

as the rheobase, had the same value within about 5 per cent at the beginning and end of a set of readings by a particular method and in which, in addition, the data of the direct current and of the condenser experiments each separately gave a smooth curve indicating that a single excitability was being dealt with   The eighteen experiments reported are from twenty which appeared to be good from the data alone   The other two when plotted appeared, however, to be mixed curves corresponding to a change from fibres of one excitability to those of another during the experiment   They were, therefore, dis

carded   Representative sets of data for different electrode separations are given in Table II

In Table I it will be seen that the condenser and D C k's are approximately equal for fourteen of the eighteen determinations   The greatest of the deviations in these cases is 8 per cent and the average is 1 5 per cent indicating that there is no systematic difference between the two values of the constant   There are four cases, however, in which the k's are very different, the condenser value being about 50 per cent greater than the D C   The time intensity curves of these cases were as good as usual so the difference cannot be attributed to poor measurement   The only explanation that can be offered is that fibres of quite different excitabilities are being stimulated in the two cases, i e , that in changing from the D C to the condenser stimuli a change was made also in the groups of fibres being stimulated   The equalities of the rheobases do not ensure that the same fibres are being dealt with for it was shown recently (Blair, 1934, Figs 4 and 8) using the data of (Blair and Erlanger, 1933) that fibres giving rise to impulses of velocities about equal might have k values very different while at the same time the relation (Blair, 1934, Equation 12)

$$\log v = -a R + \text{constant}$$

where v is the velocity, R the rheobase, and a a constant, showed that fibres with velocities about equal would have rheobases about equal It follows, therefore, as an experimental finding that fibres with very different k values may have very similar rheobases   The converse is also true so that neither does the equality of rheobases ensure that fibres of the same excitability are being used nor does the inequality of rheobases ensure that fibres of different excitabilities are being used When the rheobases are different it is probable, however, that the fibres are actually different although their excitabilities are approximately the same

Between the D C data for the preparations which gave equal k's and those which did not there was a difference which is probably significant, viz , that in the latter the constant C of Equation 2 was large and positive   This problem has been discussed previously (1932 c)   It is connected with the circumstance that a tissue with a certain k value may yield with direct current one of a family of

time-intensity curves each of which has the same shape but has for any particular intensity a displacement along the time axis which depends on C A curve with a large positive C is relatively close to the intensity axis   Or, in other words, a current of given duration need be less intense when C is large and positive than when it is small or negative   It is probably true, usually, that the fibres with the lowest rheobases have medial excitabilities (1934, Figs 4 and 8) although this is not always the case (1934, Figs 3 and 7)   It therefore appears possible that a fibre group with a given k and given rheobase, which is the easiest stimulated by condenser discharges, may not be the easiest stimulated by direct currents because it has a small or negative C, while another fibre group with larger or smaller k, which might otherwise be harder to stimulate, is made easier by virtue of its having a large C This difference in the two types of stimuli makes possible the condition that, while with condenser stimuli the curve of greater k will always lie above, i e be harder to elicit than one of smaller k when the rheobases are equal, it is not possible with direct currents to state which will be the lower unless the C values are known

In the present instance it must be supposed that rather highly excitable groups of fibres (1934, Figs 3 and 7) are the easiest excited by the condenser stimuli but they have relatively small C values compared to some others with smaller k's whose direct current curves therefore lie lower   It is, therefore, not possible to elicit a response from the same groups of fibres with both types of stimuli by using the least adequate intensities in each case   According to the results of Table I, however, it is more usual than not for the time-intensity curve of the same group of fibres to be lowest with both types of stimuli so that the same value for the excitability will be expected from either condenser or D C measurements

In view of the considerations above in regard to the four cases in Table I in which the constants k are not equal it seems legitimate to conclude from the fourteen that are equal that Equations 2 and 3 are adequate descriptions of the data even when the same value of k is common to both   The accuracy of the fit of these two equations to the data is shown in Table II

In this table are given the complete data of four experiments with different representative electrode separations   In each case the first

column gives the D C durations in seconds and the second column the corresponding voltages on an arbitrary scale In the third column are the calculated D C voltages using Equation 2 and the D C constants k and C as given The fourth column gives the ratios of the calculated

TABLE II

				Preparation b			
Duration	V D.C.	V cal.	V cal./V	c	V cond.	V cal.	V cal./V
∞	151	151	1 00	∞	151	146	0 98
0 0022	151	152	1 00	4	146	147	1 01
0 002	151	152	1 00	0 5	149	153	1 03
0 0015	155	155	1 00	0 2	160	161	1 00
0 00125	161	159	0 99	0 05	182	186	1 02
0 001	169	167	0 99	0 02	211	233	1 11
0 0008	176	177	1 01	0 01	261	291	1 11
0 0006	199	200	1 01	0 004	455	453	1 00
0 0004	237	241	1 02	0 003	531	530	1 00
0 00035	255	259	1 02	0 002	681	683	1 00
0 0003	282	284	1 01	0 001	1119	1105	0 99
0 00026	317	310	0 98	4	149		
∞	151						

D C k = 2300 or 1000 to base 10   Condenser k = 2290 C = 0 030
Resistance = 85 000 ohms   Separation of electrodes = 25 mm

				Preparation f			
Duration	V D.C.	V cal.	V cal./V	c	V cond.	V cal.	V cal./V
∞	198	198	1 00	∞	192	190	0 99
0 0022	198	198	1 00	4	195	191	0 98
0 002	200	198	0 99	0 5	199	199	1 00
0 0015	203	199	0 99	0 2	201	207	1 03
0 00125	204	202	0 99	0 05	242	243	1 00
0 001	210	207	0 98	0 02	293	298	1 02
0 0008	213	216	1 01	0 006	439	460	1 05
0 0006	239	233	0 97	0 004	559	563	1 01
0 0004	275	277	1 01	0 003	659	658	1 00
0 0003	317	324	1 02	0 002	845	850	1 01
0 00026	368	354	0 96	0 001	1349	1365	1 01
∞	198			4	199		

D C k = 3130 or 1360 to base 10   Condenser k = 3260 C = 0
Resistance = 65,000 ohms   Separation of electrodes = 20 mm.

TABLE II—*Concluded*

				Preparation *k*			
Duration	V D C	V cal	V cal/V	*c*	V cond	V cal	V cal/V
∞	102	100	0 98	∞	101	100	0 99
0 0022	101	100	0 99	4	102	101	0 99
0 002	100	100	1 00	0 5	107	106	0 99
0 0015	100	101	1 01	0 2	116	112	0 96
0 00125	103	104	1 01	0 05	131	135	1 03
0 001	109	106	0 97	0 02	178	171	0 96
0 0008	115	111	0 96	0 01	244	221	0 91
0 0006	123	122	0 99	0 006	270	281	1 04
0 0004	146	146	1 00	0 004	352	350	1 00
0 00035	157	156	0 99	0 003	408	416	1 02
0 0003	171	171	1 00	0 002	523	521	1 00
0 00026	187	187	1 00	0 001	824	902	1 09
∞	101			4	109		

D C k = 2830 or 1230 to base 10   Condenser k = 2945 C = 0 011
Resistance = 53,000 ohms   Separation of electrodes = 11 mm

				Preparation *r*			
Duration	V D C.	V cal	V cal/V	*c*	V cond	V cal	V cal/V
∞	110	110	1 00	∞	102	105	1 03
0 0022	110	113	1 03	4	110	107	0 97
0 002	110	115	1 04	0 5	111	111	1 00
0 0015	113	121	1 07	0 2	112	116	1 04
0 00125	120	127	1 06	0 05	131	137	1 04
0 001	143	137	0 96	0 02	162	168	1 04
0 0008	159	150	0 94	0 01	215	214	1 00
0 0006	174	174	1 00	0 004	308	296	0 96
0 0004	222	222	1 00	0 003	385	375	0 97
0 0003	268	271	1 01	0 002	481	494	1 02
0 00026	300	298	0 99	0 001	789	802	1 02
∞	113			4	113		

D C l = 1585 to 690 to base 10   Condenser k = 1620 C = 0 020
Resistance = 120,000 ohms   Separation of electrodes = 2 5 mm

to the observed voltages   The fifth and sixth columns contain, re-
spectively the capacities in microfarads and the voltages of the con-
denser stimuli, except in the case of the capacity marked *infinite*
which is the D C rheobase   4 microfarads can be considered as being
very nearly an infinite capacity for present purposes   The seventh

column gives the condenser voltages as calculated from Equation 3 using the condenser k as given, while the eighth column gives the ratios of the calculated to the observed condenser voltages   The methods of applying Equations 2 and 3 have been given previously (1932 a, d)   For D C the value of k using logarithms to base 10 is given in addition as it is used for calculating

Sometimes as in preparation k the same rheobase was used for calculating both the D C and condenser data   In others such as r where the D C rheobase had changed between the two sets of data a separate rheobase was used to calculate each set

TABLE III

Duration	Divergence	Capacity	Divergence
	per cent		*per cent*
∞	−0 5	∞	−0 3
0 0022	0 5	4	−1 3
0 002	0 7	0 5	0 5
0 0015	1 8	0 2	0 8
0 00125	1 3	0 05	2 3
0 001	−2 5	0 02	3 3
0 0008	−2 0	0 01	1 8
0 0006	−0 8	0 004	0 3
0 0004	0 8	0 003	0 3
0 00035	0 3	0 002	1 3
0 00030	1 0	0 001	0 5
0 00026	−1 8		

It will be seen that the observed and calculated voltages are in fair agreement throughout   In order to show a lack of systematic divergence the average divergence for each duration and each capacity is given in Table III   The D C divergences are somewhat less than those for the condensers   These condenser curves are not quite as good as can be obtained ordinarily with simpler circuits   The procedure adopted of using the same circuit for both D C and condensers makes the arrangement for the latter unnecessarily complicated   For this reason a set of voltage-capacity data obtained with a simpler circuit is given in Table IV for comparison

In Table IV are given three voltage capacity curves also from sciatic gastrocnemius preparations of the frog using a simple circuit

with just a potentiometer and the condenser and the nerve and an additional resistance in series. Three different electrode arrangements were used. In preparation 1 the electrodes were test tubes 1 cm in diameter filled with Ringer's solution. The nerve lay across the tops of the tubes, which were separated 1 cm., and through the contained solutions. The solutions were connected to circuit with silver chloride electrodes. The preparation was used with 26,800 ohms resistance in series. The resistance given in the table is the sum of this and the nerve's resistance.

Preparation 2 was stimulated in a rubber trough 1 mm. wide, 1.5 mm. deep, and 2 cm. long filled with Ringer's solution and connected to chlorinated silver wires at the ends. The preparation was shunted by 26,670 ohms in parallel as well as the Ringer's solution. The resistance given in the table is the series resistance plus the resultant resistance of the nerve and shunt.

Preparation 3 was stimulated on chlorinated silver wires in air and separated by 2 cm. It had 26,800 ohms in series and was shunted with 26,670 ohms. The resistance given is again the resultant of the combination.

It will be seen that in these cases the agreement of the calculated and observed voltages is somewhat better and that the fit is equally good with the different types of electrode.

It seems possible to conclude that Equations 2 and 3 represent time-intensity and voltage-capacity curves for the frog's sciatic nerve with as great accuracy as can be expected from the measurements. The equalities of the constants k shown here indicate, in addition, that the differential equation, Equation 1, is an accurate description of the growth of the excitatory process, because the two types of stimuli are so different in form that the fortuitous fit of the two integral equations, Equations 2 and 3, with the same constant in each would be very improbable if this were not so.

The actual mechanism of excitation may, of course, be more complex than is indicated by Equation 1. It seems necessary to conclude, however, that no matter how complex the components of this mechanism may be they work together in such a way as to give the resultant represented by Equation 1 with considerable accuracy. The bulk of the evidence points, however, to the conclusion that it is not a combina-

TABLE IV

Preparation 1				
c	V obs	V cal	V cal /V obs	
∞	0 095	0 095	1 00	
0 5	0 100	0 101	1 01	
0 2	0 100	0 102	1 02	Fluid electrodes
0 05	0 125	0 136	1 09	$k = 1,500$
0 02	0 190	0 173	0 91	Resistance $= 75\,000$ ohms
0 01	0 215	0 225	1 05	26 800 ohms in series
0 008	0 245	0 249	1 02	
0 007	0 270	0 265	0 98	
0 004	0 370	0 358	0,97	
0 003	0 435	0 428	0 98	
0 002	0 560	0 559	1 00	
0 001	0 945	0 931	0 98	

Preparation 2				
c	V obs	V cal	V cal /V obs.	
∞		1 00		
4	1 02	1 02	1 00	
1	1 07	1 03	0 96	Trough electrodes
0 5	1 13	1 12	0 99	$k = 1\,550$
0 2	1 25	1 25	1 00	Resistance $= 40\,000$ ohms
0 05	1 75	1 71	0 97	26 800 ohms in series
0 02	2 45	2 44	1 00	26 670 ohms in parallel
0 015	2 85	2 82	0 99	
0 010	3 50	3 54	1 01	
0 007	4 40	4 39	1 00	
0 005	5 50	5 45	0 99	
0 004	6 40	6 38	1 00	

Preparation 3				
c	V obs.	V cal.	V cal /V obs.	
∞	0 240	0 240	1 00	
4	0 240	0 243	1 01	
1	0 250	0 252	1 00	Silver chloride electrodes
0 2	0 265	0 281	1 06	$k = 2\,000$
0 02	0 480	0 480	1 00	Resistance $= 50\,000$ ohms
0 01	0 650	0 651	1 00	26 800 ohms in series
0 007	0 840	0 790	0 94	26 670 ohms in parallel
0 005	0 980	0 960	0 98	
0 004	1 120	1 120	1 00	
0 003	1 295	1 340	1 04	
0 002	1 80	1 79	1 00	
0 001	3 20	3 10	0 97	

tion of factors or at least not a combination of unrelated factors that is concerned   For in that case it would be expected that the applicability of Equation 1 would be very limited

Considerable evidence (1932 *a, b, c, d*) has now been presented which shows that the time-intensity and voltage-capacity curves for several different tissues of quite different excitabilities are adequately represented by Equations 2 and 3   The present evidence indicates that these equations are not only separately valid, but that both are valid on the same tissue with the same parameter *k* in each   That this factor *k* has a definite physiological significance has been shown further by the fact that it can be related fairly simply to the velocity of the nervous impulse (1934) which does not depend on the particular type of stimulus by which it is elicited and is, therefore, a purely physiological factor

SUMMARY

Data on the electrical stimulation of sciatic-gastrocnemius preparations of the frog by both direct currents and condenser discharges at the same time are discussed in relation to the validity of the differential equation

$$\frac{dp}{dt} = KV - kp$$

where p is the local excitatory process, V the stimulating current or voltage, and K and k are constants   It is concluded that the constant k is the same whether it is derived from the data of the one stimulus or the other when the same fibres are being stimulated

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A THEORY OF VISUAL INTENSITY DISCRIMINATION

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I

Necessity for New Theory

The capacity to distinguish intensity differences in the environment is an important function of sense organs   To describe this sensory capacity it has long been customary to use the fraction $\Delta I/I$, where I is one intensity and $I+\Delta I$ is that intensity which may be recognized with certainty as just higher than I

Historically the fraction $\Delta I/I$ has been associated with the Weber Fechner law, in which Fechner (1858) had originally assumed it to be constant at all intensities   However, in the last seventy five years it has been repeatedly demonstrated[1] that this assumption is not true for the eye and ear, and very likely not for the other sense organs as well   Instead, the fraction $\Delta I/I$ varies in a fairly established manner   For the ear $\Delta I/I$ decreases as I increases, the decrease is rapid at first, and then more gradual, at the highest intensities $\Delta I/I$ changes so slowly as to be almost constant (Knudsen, 1923, Riesz, 1928)   For the eye $\Delta I/I$ varies in much the same way, except that Koenig and Brodhun (1888, 1889) found the fraction to rise again at the higher intensities

Several efforts have been made to find a theoretical basis for the precise way in which $\Delta I/I$ for the visual process varies over the intensity range (Putter, 1918, Hecht, 1924 *b*, 1928, Houstoun, 1932)   These formulations all have an essential property in common   In conformity with the generally accepted data of Koenig and Brodhun they predict that as the intensity I increases, the fraction $\Delta I/I$ will

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[1] A summary of the history and data of intensity discrimination is to be found in a paper written ten years ago (Hecht 1924 *b*)

decrease steadily to a minimum, beyond which it will just as steadily increase again (see Hecht, 1934)

Very recently Wolf (1933 *a*, *b*) published measurements of intensity discrimination with bees, and Hecht and Wald (1934) with *Drosophila*, which show that the fraction $\Delta I/I$, after reaching a minimum, remains constant, and does not increase again as the intensity continues to increase   Wald and I paid particular attention to the possibility of a rise of $\Delta I/I$ at the highest intensities, but though we tested intensities even 10,000 times higher than the one at which the minimum becomes established, we found no trace of an upturn in $\Delta I/I$

Clearly the data for these two insects do not conform to previous theoretical formulation, and it becomes necessary to reconsider the basis of visual intensity discrimination

II

Basic Requirement

The measurements with *Drosophila* and with the bee were made by the method of moving stripes originally developed for the bee (Hecht and Wolf, 1929)   The insect is confronted with a visual field composed of stripes which, when moved, elicit a movement of the animal opposite in direction to the stripe displacement   The stripes are arranged to radiate any fraction of the intensity of light coming from the intervening clear spaces, and the measurements consist in finding the least difference ΔI between the light intensity I coming from the stripes and the intensity $I+\Delta I$ from the clear spaces, which will just barely elicit a response from the insect when the pattern is moved

It is important to recognize that the response of the animal follows immediately upon the movement of the pattern   A group of ommatidia, which have been exposed to the intensity I, are subjected to the higher intensity $I+\Delta I$ by movement of the pattern, and at once the insect responds [2]   Thus, though the ommatidia have been adapted

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[2] It makes no difference whether intensity discrimination is viewed in this way or in terms of a decrease in brightness ("shadow response")   In the latter case a group of ommatidia is considered as adapted to I and then suddenly exposed to $I - \Delta I$ The resulting critical equation (9) for the two formulations differs only in sign

to the intensity I, the immediate response of the insect precludes their adaptation to the higher light intensity $I+\Delta I$ The inner change in the ommatidia which initiates the events resulting in a response must therefore take place immediately when the outside light is changed from I to $I+\Delta I$ It is the properties of this initial event which form the basis of the present theory of intensity discrimination

<div style="text-align:center">III</div>

Derivation of Equations

The most general ideas about the photoreceptor system of organisms require the presence of (a) an inactive photosensitive substance which absorbs light in order to be changed by it into an active substance responsible for initiating the train of events which end in a nerve impulse, and (b) some process for maintaining a supply of the sensitive material, since otherwise it would be used up and the process would come to an end   Essentially, this is the reversible photochemical system which has served in the theoretical treatment of a variety of visual and other photosensory data (Hecht, 1934)   Obviously the photoreceptor system is more complicated than this and contains more than a reversible photochemical reaction   For our present purposes, however, it is not necessary to investigate anything beyond this very first step in the process, since by itself it yields equations which describe the data adequately

Consider a reversible photochemical system of which the sensitive material S is changed by light into the photoproducts $P, A, B, \quad$, some of which under proper conditions reunite to form the sensitive material from which they were derived   Let the total initial concentration of S be a, and the concentration of $P, A, \quad$ at the moment t be x The velocity with which the whole process will go on under the influence of light of intensity I will be

$$\frac{dx}{dt} = k_1 I(a-x)^m - k_2 x^n \tag{1}$$

where m and n represent the order of the photochemical and the dark reactions respectively, and k_1 and k_2 are their velocity constants, k_1 including the absorption coefficient   If the light continues to shine, dx/dt becomes equal to zero, and a stationary state is reached such that

the apparent concentrations of sensitive material and decomposition products remain constant   Equation (1) then becomes

$$k_1 I (a - x)^m = k \, x^n \tag{2}$$

which, when k_1/k_2 is written as K, assumes the form

$$KI = \frac{x^n}{(a - x)^m} \tag{3}$$

and is the familiar, but generalized stationary state equation

Let this photosensory system at the stationary state of equation (3) be exposed to light of intensity $I+\Delta I$ At the very first instant of exposure the velocity with which the sensitive material will be decomposed is

$$\frac{dx}{dt} = k_1 (I + \Delta I)(a - x)^m - k_2 x^n \tag{4}$$

where the concentrations are the same as during the previous stationary state, because no changes have as yet occurred   Since from equation (2), $k_1 I (a - x)^m = k_2 x^n$, we may subtract $k_1 I (a - x)^m$ and add $k_2 x^n$ to the right side of equation (4)   The result of carrying out the operations indicated, yields

$$\frac{dx}{dt} = k_1 \Delta I (a - x)^m \tag{5}$$

which says that the initial rate of photochemical decomposition on the introduction of the higher intensity to the photochemical system at the stationary state is proportional to ΔI times the concentration of sensitive material at the stationary state

For *Drosophila* and the bee, the sensory recognition of this increase in intensity occurs immediately after the increase is made   Therefore some property of the initial photochemical velocity as shown by equation (5) is the basis for the recognition of the difference between I and $I+\Delta I$ Let us assume that for the intensity $I+\Delta I$ to be distinguished from the intensity I, the initial rate of decomposition of the sensitive material on the addition of ΔI is the same no matter what the intensity I may have been [3]   This is equivalent to writing

[3] Obviously this is not the only assumption possible   Another is that the initial velocity is a function of the intensity I or of the concentration x Since I and x are related by equation (3) only the latter needs to be considered   Assume

$$k_1 \Delta I (a - x)^m = c \qquad (6)$$

where c is a constant. This constant c is formally equal to the initial rate dx/dt, this may possibly mean that in a very short time Δt, which for all practical purposes is near enough to dt, a constant photochemical change Δx has to be accomplished by the addition of ΔI, in order that the animal may just discriminate between two intensities. Since, from equation (6)

$$\Delta I = \frac{c}{k_1(a - x)^m} \qquad (7)$$

and from equation (3)

$$I = \frac{x^n}{K(a - x)^m} \qquad (8)$$

we get, by dividing (7) by (8) and remembering that $K = k_1/k_2$,

$$\frac{\Delta I}{I} = \frac{c}{k_2} \frac{1}{x^n} \qquad (9)$$

as a description of $\Delta I/I$ in terms of the general ideas we have just considered.

In order to test these ideas with data it is necessary to try specific values for m and n in equations (8) and (9). In this way we eliminate x and derive $\Delta I/I$ as a function of I. It is in this form that the data exist. The simplest case is when $m = n = 1$, that is when both the

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then that to distinguish $I + \Delta I$ from I the initial velocity dx/dt instead of being constant is proportional to x or to x alone. In the first case, equation (5) becomes $k_1 \Delta I(a-x)^m = hx$ where h is a constant. Thus $\Delta I = hx / k_1(a-x)^m = hI/k_1$ When both sides are divided by I this gives $\Delta I/I = h/k_1$ which we know is not true experimentally. In the second case equation (5) becomes $k_1 \Delta I(a-x)^m = hx$ Solving for ΔI and dividing by I we get $\Delta I/I = h/k_2 x^{-1}$ as the general expression corresponding to (9). Assuming $m = n = 1$ makes $\Delta I/I = h/k_2$ which again is not true experimentally. Assuming $m = n = 2$ and $m = 1$ $n = 2$ gives expressions for $\Delta I/I$ which are the same as (13) and (15) but without the square exponent on the right side. Plotted as $\log \Delta I/I$ against $\log I$ these expressions yield curves whose slopes are exactly half of those given by equations (13) and (15) *and by the measurements* In other words they fail completely to describe the data. It may be added that quite a variety of assumptions have been similarly tested and have been discarded.

light and the dark reactions are monomolecular   Substituting these values for m and n in (8) and solving for x, we get

$$x = \frac{a}{1 + 1/KI} \tag{10}$$

which, when substituted in (9), gives

$$\frac{\Delta I}{I} = \frac{c}{ak_2}\left(1 + \frac{1}{KI}\right) \tag{11}$$

the desired expression for intensity discrimination   When equation (11) is plotted as log $\Delta I/I$ against log I, the form of the function is independent of the constants K and c/ak_2 The value of K controls the position of the curve on the axis of abscissas, that of c/ak_2 on the axis of ordinates   Putting $K = 1$, and $c/ak_2 = 1$ gives the curve marked 1,1 in Fig 1

When $m = n = 2$, equation (8) gives

$$x = \frac{a}{1 + 1/(KI)^{\frac{1}{2}}} \tag{12}$$

which when substituted in (9) yields

$$\frac{\Delta I}{I} = \frac{c}{a^2k_2}\left(1 + \frac{1}{(KI)^{\frac{1}{2}}}\right)^2 \tag{13}$$

an expression similar in many respects to (11)   Like (11) it may be plotted in logarithmic form as the curve marked 2, 2 in Fig 1, in which case its precise shape is independent of the constants K and c/a^2k_2

The unsymmetrical case of $m = 1$ and $n = 2$ gives the following value of x from equation (8)

$$x = \frac{(4aKI + K^2I^2)^{\frac{1}{2}} - KI}{2} \tag{14}$$

which may then be put into equation (9)   This becomes transformed into

$$\frac{\Delta I}{I} = \frac{c}{k}\frac{4}{[(4aKI + K^2I^2)^{\frac{1}{2}} - KI]^2} \tag{15}$$

an equation which may be plotted logarithmically (the curve marked 1,2 in Fig 1) in the same way as (11) and (13)   The unsymmetrical

case when $m = 2$, $n = 1$ is not developed here because none of the data to be considered are exclusively fitted by the resulting equation In all cases the total concentration of sensitive material is put at 100 per cent, thus $a = 1$
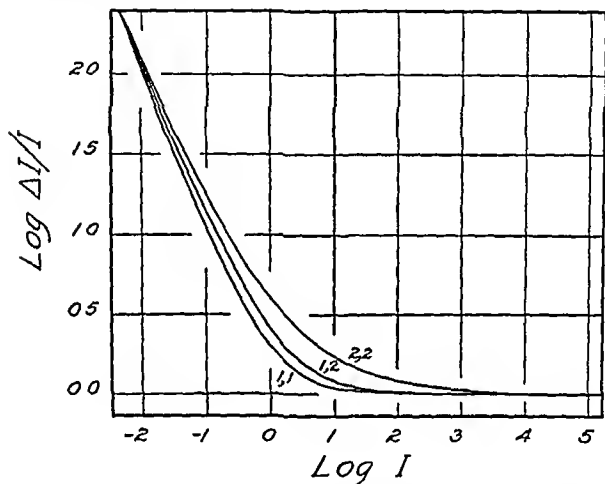
FIG 1 The relation between $\Delta I/I$ and I in terms of the theoretical equations Drawn on a logarithmic grid, the curves have a shape which is independent of any constants in the equations but is determined by the order of the light and dark reactions of the photosensory system, as shown by the numbers attached to the curve The first number gives the order of the light reaction, the second of the dark reaction Curve 1,1 is from equation (11) Curve 1,2 from equation (15), and Curve 2,2 from equation (13)

The curves derived from equations (11), (13), and (15), and shown in Fig 1 are obviously of the same family The two corresponding to $m = n = 1$ and $m = 1$, $n = 2$ are very similar, and a decision as to which applies to a set of data can be made only when the measure ments are very precise and cover the greater part of the curve Ap parently their common value of m determines the specific properties

of the resulting $\Delta I/I$ curve, because the one corresponding to $n_e = n_r$ = 2 is distinctly different from both because of the gradual way in which the descending portion joins the horizontal portion of the curve As a result there is little difficulty in deciding whether a set of data is fitted by it or by the other two curves [4]

<div align="center">IV</div>

Insects

The theoretical behavior of $\Delta I/I$ in all three curves of Fig 1 resembles the actual behavior of $\Delta I/I$ for insects   The data for *Drosophila* are shown in Fig 2   Through them is drawn the curve for $\Delta I/I$ in accordance with equation (11)   The fit with this equation is slightly though not decisively better than with equation (15), equation (13), however, is definitely ruled out   The choice of equation (11) instead of (15) rests on the fact that the relation of the visual acuity of *Drosophila* to intensity is described with fine precision by an equation of the form $KI = \nu/(a - \nu)$, and not at all by $KI = \nu^2/(a - \nu)$ (Hecht and Wald, 1934)   Fig 2 shows that the present theoretical formulation for intensity discrimination accurately describes the data of *Drosophila*

The first measurements of intensity discrimination of the bee made by Wolf (1933*a*), and reproduced in Fig 3 by black circles, are too scattered for a critical choice among the theoretical curves   The later measurements (Wolf, 1933*b*), shown in Fig 3 by clear circles, are smoother, more numerous, and more critical than the first ones Of the three theoretical curves, only the one from equation (13) can be drawn through them   The exactness of fit is obvious   Because of the adequacy of equation (13) for the later data, the same curve is drawn through Wolf s earlier data, though the measurements themselves give no warrant for a choice among the three equations

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[4] The relations between ΔI and I in the above equations may be plotted in a variety of ways   The logarithmic form here used was chosen because it showed so easily which of the three equations (11), (13), and (15) fit a set of measurements, The curves of the equations are drawn on individual sheets of coordinate paper The data, plotted on the same scale on a similar sheet are compared with the three curves by transmitted light   Because of the logarithmic plot the theoretical curve may be moved in all directions—provided only that the axes of the two graphs are kept parallel—and clearly demonstrates when it does or does not fit a given set of data

V

Mya

It is not surprising that the present theoretical formulation fits the data for *Drosophila* and for the bee The method of experimentation, which relies on immediate changes produced by the added light, is
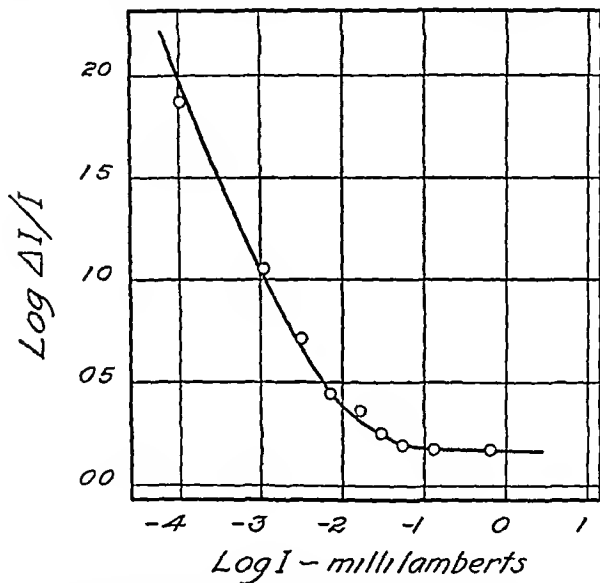
FIG 2 The data of intensity discrimination of *Drosophila* The theoretical curve is from equation (11) and is the same as 1 , 1 in Fig 1

responsible for equation (4) which, with equation (6), are the critical equations of the theory It is revealing to examine the other avail able data of visual intensity discrimination in the light of the new ideas

The measurements of intensity discrimination with *Mya* made ten

years ago (Hecht, 1924 a) also record immediate responses to an increase in the intensity of illumination *Mya* was first adapted to a
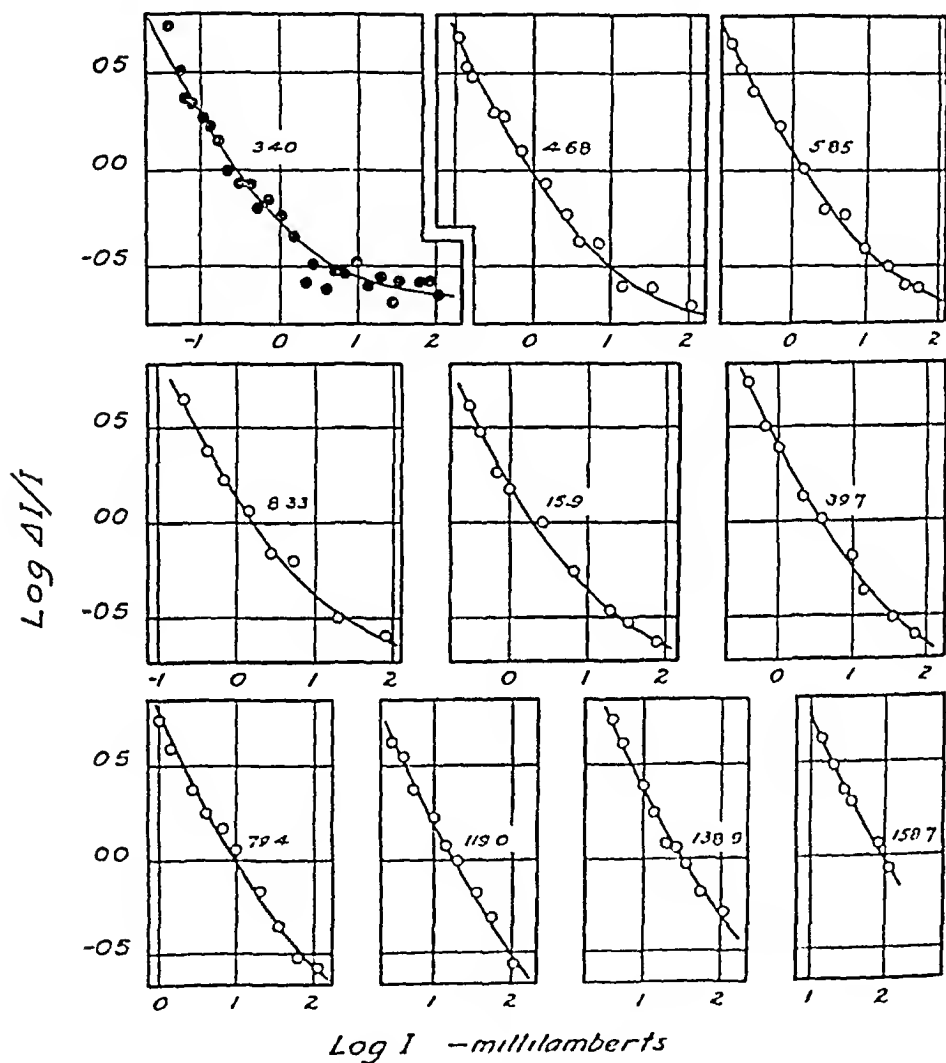
Fig 3 Wolf's measurements of the intensity discrimination of the honey bee The black circles are the data from the first paper, the plain circles from the second paper The numbers attached to the curves are the visual acuities multiplied b 1000 and are inversely proportional to the size of the stripes used for the measurements The same theoretical curve is drawn through all the data, it is Curve 2, 2 of Fig 1 and represents equation (13)

given intensity then an additional intensity w   ded and the reaction time of the animal to the

This was done for three different values of the added intensity for each of seven adapting intensities   By plotting reaction time against added intensity for each adapting intensity, it was possible to derive graphically the intensity required to be added to each adapting intensity in order for *Mya* to respond at the end of a specific reaction time   Fig 4 shows the relationship between the added intensity ΔI and the
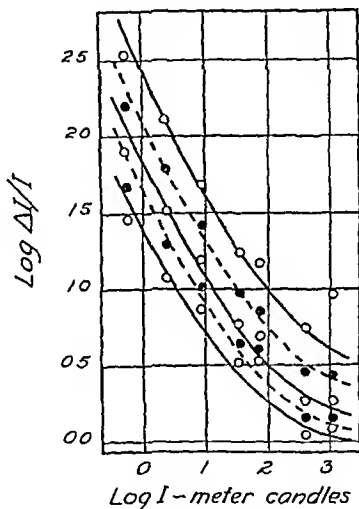
Fig 4 Intensity discrimination of the clam, *Mya*   The clear circle so obviously off is an extrapolated value   The curves are all the 2 2 curve from Fig 1 and equation (13)

adapting intensity I required for five different reaction times   As before the data are in the form of $\log \Delta I/I$ against $\log I$, from which it appears that the relationship is the same for all reaction times

The experimental procedure with *Mya* records the immediate response of the animal   We may therefore expect the present theoretical formulation to apply   The curves drawn through the data in Fig 4 are all from equation (13), which is the only equation of the

three to fit them   To derive equation (13), $n = m = 2$ That n = 2 for *Mya* is not surprising since the dark reaction has long been known to be bimolecular (Hecht, 1918)   The fact that the purely photochemical reaction is also of the second order emerges for the first time from this theoretical treatment

The data for *Mya* in Fig 4 reach just to the tantalizing minimum of the theoretical curve   The added illuminations ΔI at this point are of the order of 10,000 meter candles, which were the highest intensities available at the time   It is obviously necessary to determine whether $\Delta I/I$ remains constant at still higher intensities, and experiments will have to be devised in which more powerful beams of light can be used with this animal

<div align="center">VI</div>

<div align="center">

Human Eye

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The proposed theory of intensity discrimination has been successful in accurately describing the data of *Drosophila*, of the bee, and of *Mya* Can a similar theory be applied to the human eye?   On first impulse the answer appears to be no   Measurements of visual intensity discrimination are usually made with a bipartite test field of which one side has an intensity I and the other an intensity $I+\Delta I$ The procedure seems to involve the simultaneous and complete adaptation of the correspondingly juxtaposed retinal areas to their respective intensities,[6] whereas our critical equations depend on the initial effect

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[6] The formulation of intensity discrimination made ten years ago (Hecht, 1924 *b*, 1928) rests on this basis   It was then assumed that the eye will just discriminate between two intensities when their corresponding stationary states differ by a constant amount of photoproducts   Such a formulation predicts a rise in $\Delta I/I$ at high intensities (*cf* especially Hecht, 1934)   It seemed to apply to *Mya* because the data extend only to the minimum, as Fig 4 shows   It also seemed to describe the data of Koenig and Brodhun when the usual assumption is made that the rods and cones function independently, and if the data are corrected for variable pupil   Since then, the pupil correction had to be discarded, first because Koenig and Brodhun apparently used an artificial pupil (Koenig, 1897, footnote), and second because Schroeder (1926) and Stiles and Crawford (1933) found such corrections to be inadequate   Furthermore, as will presently be apparent, the upturn of $\Delta I/I$ at high intensities is probably spurious, and does not appear under properly controlled conditions

of the added intensity ΔI to produce the significant difference between the two just perceptibly different intensities

It is possible, however, that this antithesis is more apparent than real. In making the measurements, the eye looks at and becomes adapted to the intensity I which prevails on both sides of the field. Then the intensity on one side is raised slightly, and a judgment is made as to whether there has been a perceptible increase in brightness. The procedure is continued until the minimum increase is found which is clearly recognizable not only at once but on continued examination. It is to be noted, however, that any continued study of the field involves the usual and persistent eye movements which expose fresh portions of the retina to the higher intensity, while the general state of adaptation of the retina still corresponds to the lower intensity. If this is correct, then recognition of ΔI may involve initial rather than final effects even with the human eye. Certainly no harm can be done by testing the data in terms of the equations

The existing material on the human eye is quickly summarized. The first quantitative measurements were made by Aubert in 1865, and effectively disposed of Fechner's idea that $\Delta I/I$ is constant. Twenty five years later Koenig and Brodhun measured the values of $\Delta I/I$ over the whole range of intensities for white light and for six portions of the spectrum. Like Aubert they found that as I increases $\Delta I/I$ decreases steadily to a minimum, at still higher intensities, beyond those at which Aubert worked, they found $\Delta I/I$ to increase again. In 1918 Blanchard repeated the measurements with white light up to but not including very high intensities, and found $\Delta I/I$ to decrease steadily in much the same way as had Aubert. Since 1924, when it was shown that, plotted as $\Delta I/I$ against log I, the data of these four observers could be superimposed on a single graph (Hecht, 1924 b), there have been some minor additions to the data. Holladay (1927), as part of a study of glare, determined $\Delta I/I$ for a portion of the intensity range, and secured data which resemble those of Koenig and Brodhun, without, however, going to high enough intensities to confirm or deny the rise of $\Delta I/I$ found by them. Over a small range of high intensities Lowry (1931) measured $\Delta I/I$ and secured values which serve to extend Blanchard's data. Finally, Houstoun (1932) has recently published measurements of $\Delta I/I$ with untrained observers

Some of these show an increase in $\Delta I/I$ at high intensities, but the measurements as a whole are very irregular, and such details of the procedure as are given do not inspire confidence in the data   The increase in $\Delta I/I$ at high intensities will be dealt with more explicitly in a few pages

The common practice of plotting $\Delta I/I$ arithmetically against log I tends to compress the low values of $\Delta I/I$ into a small space at the bottom of the graph   Therefore, for reexamining the available data with a view to ascertaining their relationship to the present formulation, they have been plotted as log $\Delta I/I$ against log I

The data for Aubert's eye are shown in Fig 5   The points are single measurements, and are therefore fairly rough, but their significance is unequivocal   The data clearly range themselves into two parts As the intensity increases, $\Delta I/I$ first decreases sharply and then reaches a plateau which continues for at least one log unit, after which $\Delta I/I$ again drops sharply   The two parts are a demonstration of the duplicity theory, in terms of which the low intensity limb represents the function of the rods, and the high intensity limb that of the cones The two sections of the data are fitted by the equations developed in the present theoretical treatment   The low intensity data are not good enough for a choice between equations (11) and (15), but they are adequate tentatively to rule out equation (13)   Equation (15) has been drawn through them, because the dark adaptation of the rods indicates that the dark reaction is probably bimolecular (Hecht, 1934)   The data for the high intensity cone portion are too limited for a critical choice among equations (11), (13), and (15)   The curve drawn through them is from equation (13), but this is on the basis of the other measurements to be considered

Leaving Koenig and Brodhun's white light data to one side for the moment, let us examine Blanchard's data shown as plain circles in Fig 6   They do not cover quite so large a range as Aubert's, but the points are smooth, and precisely determined   It is striking that these data also break by themselves into two portions   The curve through the rod portion is again from equation (15), though the curves from (11) and (13) are indistinguishable from it for the very small portion covered by the few points   The cone portion containing only six points may be supplemented by Lowry's measurements made in the

same laboratory thirteen years later, which cover the higher intensities For the intensities at which they overlap, Lowry's $\Delta I/I$ values are slightly lower than Blanchard's, they have accordingly been raised 0 15 log units along the ordinates in Fig 6, so as to be continuous with
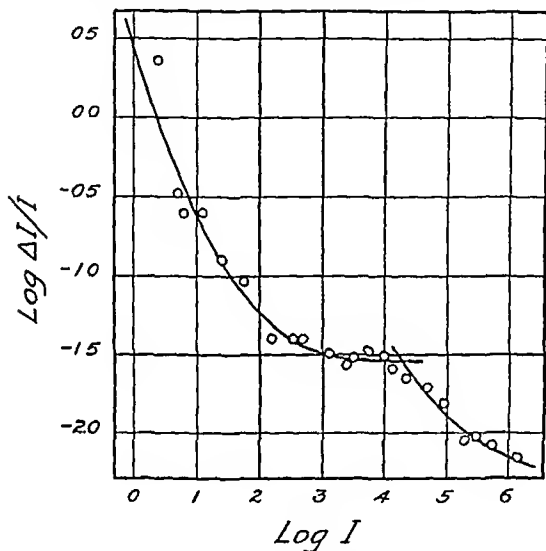
FIG 5 Aubert's measurements of the intensity discrimination of his own eye The intensities are those given by Aubert Judging by his description of the experiments they should be divided by 500,000 to convert them into millilamberts The data strikingly break into the two sections representing rod and cone functions The curves are from Fig 1 the one at low intensities representing equation (15) and the one at high intensities equation (13)

Blanchard's The curve through the data is from equation (13) and is the only one of the three equations which passes through the points

These measurements were all made with large test fields and with white light, and show the separate presence and function of the rods

and cones   Fig 7 contains the data of Koenig and Brodhun for the
red, orange, and yellow portions of the spectrum using a rectangular
field 4° ⨯ 6°   Since the extreme red light of the spectrum even at low
intensities is more effective for the cones than for the rods, it is not
surprising to find that the points for 670 mμ lie on one continuous
curve and show no trace of the break so strikingly present with white
light   The data are fitted only by equation (13), which is the one
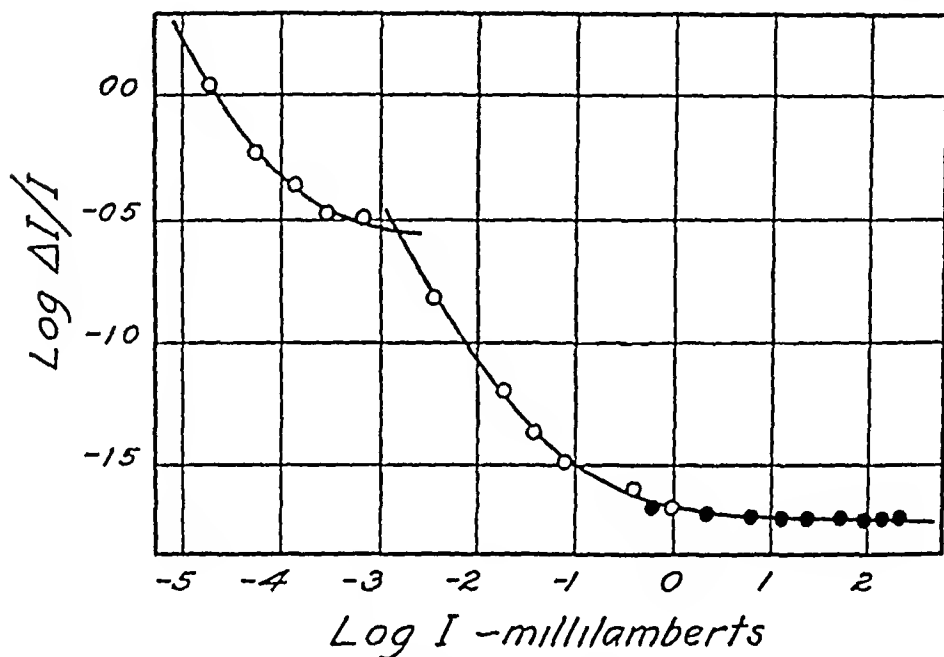
FIG 6 The measurements of Blanchard are the plain circles, those of Lowry
are the black circles   The curves are the same theoretical ones as for Aubert's
data in Fig 5   Note here too the natural breaking of the data into two sections
indicative of rod and cone functions

used for the cone portions of Figs 5 and 6   The data for 605 mμ
and for 575 mμ show the usual discontinuity and the separate presence
of rod and cone function   As would be expected from the relative effec-
tiveness of 605 and 575 mμ at low intensities for rods and cones (Hecht
and Williams, 1922, Kohlrausch, 1922, Hecht and Verrijp, 1933) the
rod portion for 575 mμ is larger than for 605 mμ The cone portions
of 605 and 575 mμ are fitted only by the same equation (13) which has
been used for all the cone data

Judged by the available data so far presented, the theory seems to be successful in describing intensity discrimination for the human eye There are two things, however, which need to be discussed, both con-
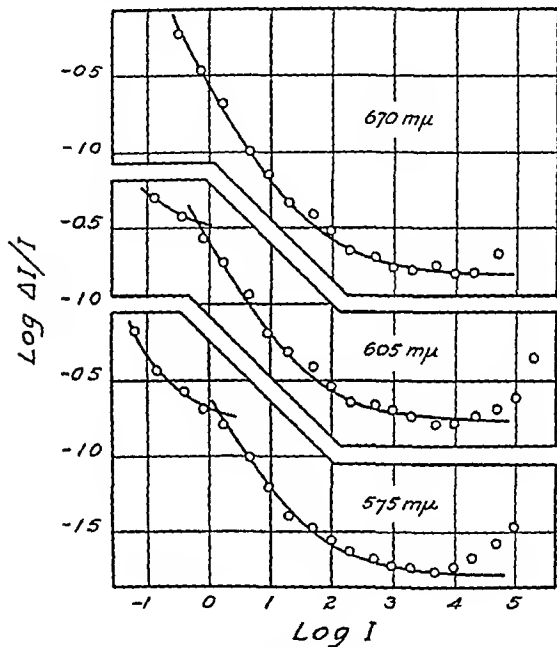
Fig 7 The data of Koenig and Brodhun for Koenig s eye for red orange, and yellow spectral light The red data are continuous and show only cone function, whereas the orange and yellow show increasing amounts of rod function The curves are theoretical and are from equation (15) for the rod section and from (13) for the cones

cerning the data of Koenig and Brodhun The first is the increase in $\Delta I/I$ at high intensities found by these workers, the rise is present in the three sets of spectral data shown in Fig 7, and in the white light

data of the same observers shown in Fig 8   If we compare these data with those in Fig 6 it is apparent that Lowry's $\Delta I/I$ values are practically constant at brightnesses easily fifty times as high as those at which Koenig and Brodhun's $\Delta I/I$ values have already risen sharply   Their rise is therefore suspicious and may be due to the way the measurements were made   Dr Jacinto Steinhardt, who has been studying the intensity discrimination of his own eye, has informed me that the appearance of this rise in $\Delta I/I$ depends on the illumination surrounding the test field and on the adaptation of the eye   The better the adaptation, the less evident is the upturn, and when adaptation is complete, there is little or no upturn for the high intensities, especially when there is a good sized field surrounding the test area   Koenig and Brodhun had no surround to their test field [6]

The second thing about Koenig and Brodhun's data is not so easily understandable   Fig 8 shows their measurements with white light, and it is apparent that these resemble neither Aubert's nor Blanchard's, nor indeed their own data with orange and yellow light, particularly the latter which usually produces effects very similar to white light   They show no clear division into two parts, and though the points fall around two theoretical curves such as are shown in Fig 6, they are not really fitted by them   Holladay's data, though few and scattered, resemble Koenig and Brodhun's measurements more than Aubert's and Blanchard's   Even more puzzling are Koenig and Brodhun's blue and violet data   Since at low intensities the rods are more sensitive than the cones to blue light, it might be expected from what has been found with red, orange, and yellow light in Fig 7, that these data would show a large rod portion and only a small cone portion Yet the data show only a continuous and shallow decrease in $\Delta I/I$ with no indication of a break   Nor do the points fall on any of the three theoretical curves

There is the obvious possibility that at those intensities where both

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[6] Mr J Gould of the National Physical Laboratory in discussing Houstoun's recently reported measurements of $\Delta I/I$ records an experiment, which he has kindly demonstrated to me, which shows simply and convincingly that the rise in $\Delta I/I$ at high intensities is "entirely factitious and depends on the degree of adaptation to each field-intensity which prevails when the observation is made" (p 180, Report of a joint discussion on vision, Physical Society, London, 1932)

rods and cones are functional, their effects summate to give values of $\Delta I/I$ lower than either would give alone, this would account for the absence of inflection point and of clearly separated rod and cone portions But there remains the fact that Aubert's data, and Blanchard's data, and more emphatically Koenig and Brodhun's own red, orange, and yellow data show no such summation, but show instead a clear inflection point indicating the passage of the function from rods to cones Thus Koenig and Brodhun's measurements are not consistent
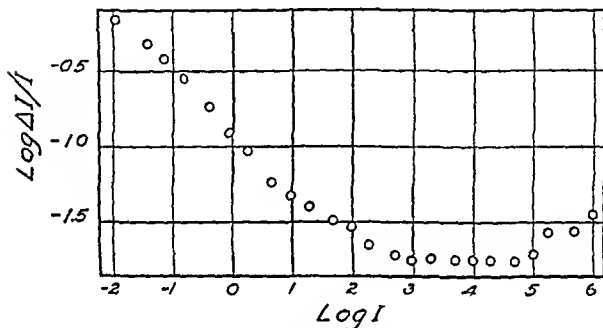
FIG 8 Koenig and Brodhun's data for white light (Koenig's eye) The intensity is in Koenig's original units Though there is an indication of an inflection between the fourth and fifth point from the left, it is slight and distinctly different from that present in Koenig's own yellow data in Fig 7 and in Aubert's (Fig 5) and Blanchard's data (Fig 6) Moreover none of the theoretical curves of Fig 1 really fit the points

because their data with yellow, orange, and red light present a significantly different appearance from their data with white, blue, and violet light Some condition in the procedure must be responsible for the difference, but unfortunately Koenig and Brodhun published only the scantiest technical details, and leave one at a loss to know what this condition is

Steinhardt's measurements, soon to be published, confirm these doubts Steinhardt measured $\Delta I/I$ over the whole visual intensity range for a variety of test field sizes His results, obtained over several

years, are essentially uniform, and he has kindly permitted me to refer to them here   For white light and test areas larger than 2° the data, without exception, fall on a double curve similar to the data of Aubert and of Blanchard, while for smaller, foveally fixated areas, they always form single curves like those of Koenig and Brodhun with light of 670 mμ   Fig 9 shows two examples   The upper data are for white light and a test area 56′ in diameter, having a large surround in order to
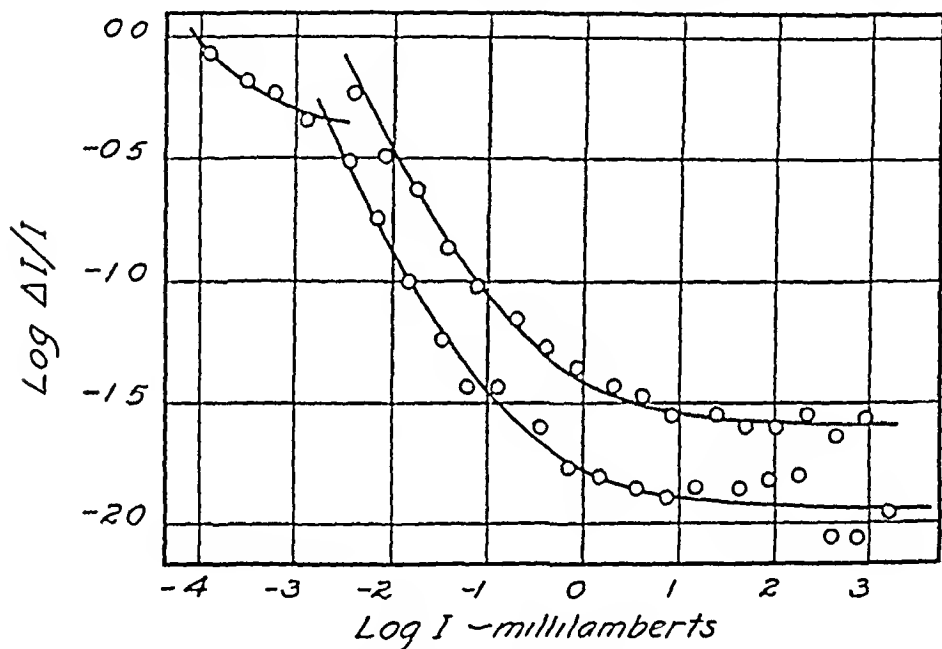
Fig 9 Steinhardt's measurements with white light   The upper data are with a field 56′ in diameter, the lower with a field 3°44′ in diameter   The upper data show only cone function and are described as usual by the curve from equation (13)   The lower data show both rod and cone function, the curve through the former is from equation (15), while through the latter it is from (13)

maintain the eye as a whole at the intensity of the measurements   This size of test field falls entirely within the rod-free area of the fovea   The data are continuous and show no inflection point   The only curve which passes through the points is from equation (13) and is the one which has been used with the other cone data   The lower data in Fig 9 were chosen because they are with a field size of the same order of magnitude as Koenig and Brodhun's   They happen to be one of Steinhardt's earliest set of measurements, but like all others, they

clearly resemble the white light data of Aubert and of Blanchard, and not those of Koenig and Brodhun The curves drawn through them are as usual from equation (15) for the short piece at low intensities, and from equation (13) for the rest of the data

It is no pleasure to question even a portion of the data of such eminent investigators as Koenig and Brodhun But these comparisons show that those of Koenig and Brodhun's data which agree with the measurements of other investigators are adequately described by the theory, whereas those which are not fitted by the theory are not corroborated by other investigators both before and after Koenig and Brodhun

SUMMARY

1 A theory of visual intensity discrimination is proposed in terms of the photochemical events which take place at the moment when a photosensory system already adapted to the intensity I is exposed to the just perceptibly higher intensity $I+\Delta I$ Unlike previous formulations this theory predicts that the fraction $\Delta I/I$, after rapidly decreasing as I increases, does *not* increase again at high intensities, but reaches a constant value which is maintained even at the highest intensities

2 The theory describes quantitatively the intensity discrimination data of *Drosophila*, of the bee, and of *Mya*

3 With some carefully considered exceptions the intensity discrimination data of the human eye fall into two classes those with small test areas or with red light, which form a single continuous curve describing the function of the retinal cones alone, and those with larger areas, and with white, orange, and yellow light, which form a double curve showing a clear inflection point, and represent the separate function of the rods at intensities below the inflection point and of the cones at intensities above it

4 The theory describes all these data quantitatively by treating the rods and cones as two independently functioning photosensory systems in accordance with the well established duplicity idea

5 In terms of the theory the data of intensity discrimination give critical information about the order of both the photochemical and dark reactions in each photosensory system The reactions turn out to be variously monomolecular and bimolecular for the different animals

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RELATION BETWEEN BIRTH WEIGHT AND LITTER SIZE IN MULTIPAROUS MAMMALS

By E V ENZMANN and W J CROZIER

(*From the Laboratory of General Physiology, Harvard University, Cambridge*)

I

It has long been known that there exists a negative correlation be tween the number of young in a new born litter of multiparous mam, mals and the birth weight of the young This relationship has been shown to hold for rabbits (Kopeć, 1924, Hammond, 1921), for rats (King, 1915, Stotzenberg, 1915), for guinea pigs (Wright, 1922, Ibsen, 1928), and for albino mice (Bluhm, 1929) In man also the relation ship seems to hold, Strassmann (1903) reported that triplets are about 5 cm shorter at birth than single born

Bluhm (1929), Wishart and Hammond (1933) and other observers have shown that the birth weight is influenced by a variety of factors weight, age, and state of nutrition of the mother, and so forth The customary method of estimating the influence of each of the factors upon birth weight has been to calculate the coefficient of correlation between each variable and the birth weight The coefficient of cor relation between the average birth weight of an individual and the size of the litter of which it is a member has been given as

$$r = -0\,37 \pm 0\,015 \text{ for the albino mouse, by Bluhm}$$
$$r = -0\,66 \qquad \text{for the guinea pig by Wright}$$
$$r = -0\,62\,(0\,60) \qquad ' \qquad ' \qquad ' \text{, by Eaton}$$
$$r = -0\,866 \pm 0\,034 \text{ and}$$
$$r = -0\,718 \pm 0\,043 \text{ for two races of rabbits, by Kopeć}$$

Although attempts have not been wanting to arrive at an under standing of the mechanism responsible for this high correlation be tween birth weight and litter size, no exact formulation of the rela tionship has been given In the present experiments an empirical

equation has been obtained, and its bearing upon the possible mechanism responsible for the correlation is discussed

II

The material was obtained during an investigation of prolonged gestation in albino mice (Enzmann, Saphir, and Pincus, 1932)   It consisted of a large series of healthy young females of the Bagg albino strain   The animals were of the best breeding age, roughly between the 15th and 30th week of age   The line has been inbred for more

TABLE I

Relation between the litter size and the average weight of the whole litter in albino mice   Present experiment

Litter size N	Litter weight W
	gm
1	—
2	—
3	4 89
4	5 11 ± 0 122
5	7 70 ± 0 101
6	8 70 ± 0 128
7	10 22 ± 0 186
8	10 96 ± 0 119
9	12 11
10	12 65
11	13 50

than twenty-eight generations   The new-born young of each mother were weighed as early as it was convenient, which was always within 12 hours after they had been born   New-born mice which had been fed by the mother were not included   The litter was weighed as a whole and the results with each group were averaged

Table I shows the birth weights of litters ranging in size from 3 to 11 young in a litter   The curve resembled that of a power function Plotting logarithms of average birth weights ($\log W$) against logarithms of litter size ($\log N$), a straight line is obtained (Fig 1)

The series of animals used here is relatively small (414 individuals) To test the relationship, data furnished by other authors were ex-

amined   Bluhm (1929) gives figures on the birth weights of over 18,000 albino mice, Marshak (unpublished data) obtained birth weights on a large number of a strain of chocolate mice bred in this
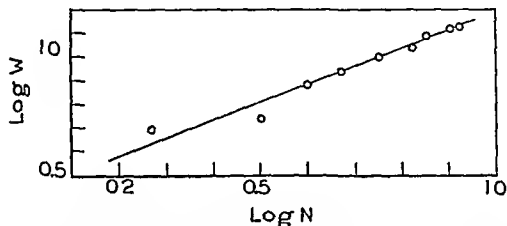
FIG 1 Relation between the average weight of whole litters and the litter size in multiparous mammals   Data on albino mice   Present experiment

TABLE II

Relation between the litter size and the average weight of whole litters in different strains of mice   Data by Gates (1925) Bluhm (1929), and Marshak (unpublished)

Litter size N	Birth weight W (Gates)	Birth weight W (Bluhm)	Birth weight W (Marshak)
1	—	1 29	—
2	3 74	2 76	—
3	5 34	3 99	—
4	6 60	5 04	5 64
5	7 10	6 15	7 46
6	8 52	7 14	8 49
7	9 87	8 12	9 41
8	10 64	8 96	10 55
9	11 43	10 08	11 24
10	13 40	10 70	12 36
11	14 63	11 88	14 43
12	15 84	—	—

laboratory, and Gates (1925) has given birth weights of another strain of mice   These data were recalculated and are given in Table II and in Fig 2   On the whole, these data give a much better fit
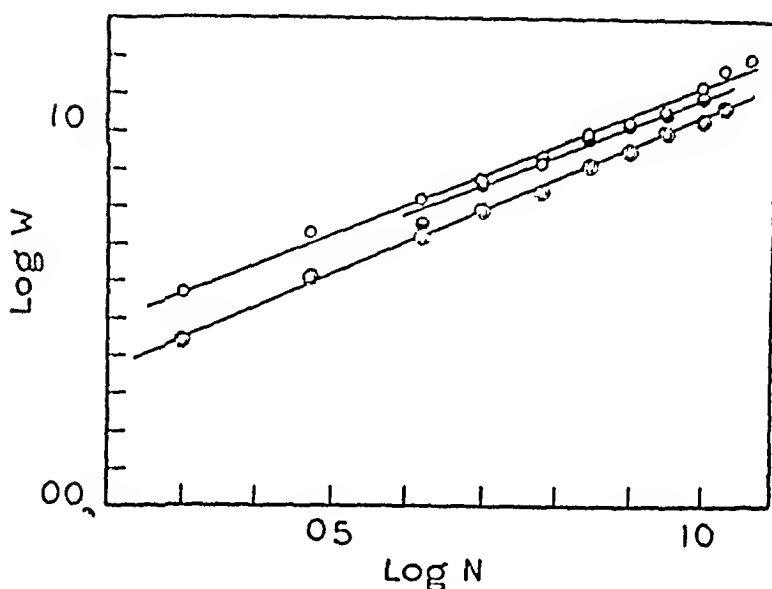
FIG 2 Relation between the average weight of whole litters and the litter size in multiparous mammals Data from Bluhm (1929), Gates (1925), and Marshak (unpublished), on various strains of mice

The double circles represent Bluhm's data, the single circles Gates' data, and the half-filled circles Marshak's chocolate mice

TABLE III

Relation between the litter size and the average birth weight of whole litters of different species of rodents and of different strains of the same species Data by Kopeć (1924) on rabbits, by Wishart and Hammond (1933) on rabbits, and by Minot (1891) on guinea pigs

Litter size, N	Himalayan rabbits (Kopeć)	Silver rabbits (Wishart and Hammond)	Rabbits, C strain (Wishart and Hammond)	Rabbits I strain (Wishart and Hammond)	Guinea pigs (Minot)
	Birth weight, W	W	W	W	W
1	48 7	75 0	90 5	55 4	85 5
2	94 2	—	176 8	100 6	157 0
3	129 6	—	228 3	141 0	204 0
4	154 0	150 8	260 0	172 8	256 4
5	184 5	223 5	327 5	206 5	299 0
6	200 4	240 6	348 6	239 4	373 2
7	227 5	265 3	365 4	275 8	396 7
8	—	291 2	409 6	324 0	417 6
9	—	—	459 9	—	—
10	—	—	482 0	—	—
11	—	—	486 2	—	—
12	—	—	542 4	—	—
13	—	—	487 5	—	—

than do our own data, chiefly due to the fact that they are based on larger series

Minot (1891) published birth weights of guinea pigs, Kopeć (1924) gave birth weights for several races of rabbits, and Wishart and Hammond (1933) have published birth weights on three races of rabbits These data have been recalculated and are given in Table III and Fig 3
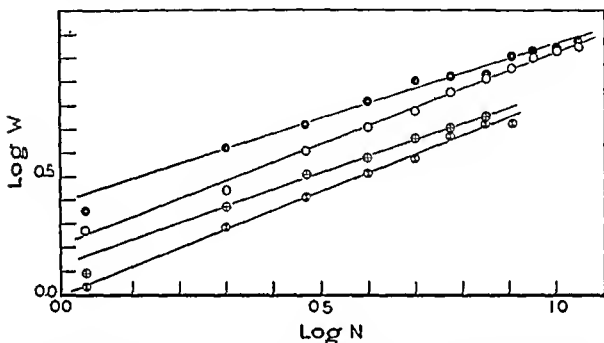
FIG 3 Relation between the litter size and the average weight of whole litters, for different species of rodents and different strains of the same species Data by Bluhm (1929) on mice, by Kopeć (1924) on rabbits, by Wishart and Hammond (1933) on rabbits, and by Minot (1891) on guinea pigs

The black circles represent the birth weights of a strain of rabbits from Wishart and Hammond s paper the circles enclosing white crosses give the birth weights of a strain of rabbits studied by Kopeć, the circles enclosing slanting crosses represent data on guinea pigs by Minot, and the white circles show the data on Bluhm's mice In order to save space the scales for different species on the ordinate were telescoped The scale marked zero at the origin is for the mice for the guinea pigs the origin of the ordinate should read 1 9 and for the rabbits 1 6

Our own results as well as those given by other observers show that the relation between the average birth weight of multiparous mammals and the litter size may be expressed by the equation

$$\Delta W / W = K(\Delta N / N)$$

or,

$$W = N^K + C$$

where W stands for the average weight of a whole new-born litter, N for the litter size, and C and K are constants

We did not include in our data (Table I and Fig 1) litters of less than three young   Very small litters are rare and are in many cases due to embryo mortality   One may therefore expect that the figures expressing the average birth weights of very small litters should be too low, this is indeed the case, as most of the data on mice taken from the literature show   In rabbits and guinea pigs the conditions are similar

III

Several theories have been proposed to account for the correlation between the litter size and birth weight of multiparous mammals, but unfortunately none in its present form is capable of explaining all the facts

Minot (1891) proposed that the differences in birth weight are due to differences in the time of gestation   This theory explains to a large extent the correlation between birth weight and litter size in guinea pigs, but fails if applied to rabbits and mice   In these forms one may obtain large litters in a pregnancy of short duration and small litters born after a comparatively long pregnancy   For this reason the theory of Keilmann (1891) and others, that internal pressure brings about parturition, meets with similar difficulties   The tension upon the uterine muscles depends upon the size and weight of the embryos enclosed in the uterus   If parturition depended upon reaching a threshold tension of the uterine muscles the length of pregnancy should be roughtly inversely proportional to the litter size   This is certainly not the case, although there is a slight correlation between the length of pregnancy and the litter size (Wishart and Hammond, 1933, *et al*)

It might also be assumed that the birth weight is a function of the size of the placenta   There is some evidence for this view   Draper (1920) describes a case where one uterine horn contained one embryo while the other horn held two   The placenta of each of the twins was lighter than the placenta of the single individual   The junior writer observed in some cases of large litters in mice that the crowding led to partial fusion of placentae   One of the objections to this

theory is that it assumes a constancy of the efficiency of the placenta as a nutritive organ   There are indications (Enzmann, unpublished data, Wishart and Hammond (1933)) that this is not the case in either the mouse or the rabbit

Spiegelberg (1891) advanced the view that the embryos in the uterus release a substance, hormonal in nature, which induces parturition when its concentration reaches a definite limit   The same objections which were brought forward against the uterine tension theory also apply to this explanation   The current view is that the length of pregnancy is conditioned by ovarian hormones (Ancel and Bouin, 1912, Hammond, 1917, Schafer, 1917, Wishart and Hammond, 1933, *et al*)   The time course of pregnancy may therefore be entirely independent of the number of young carried

Bluhm (1929) discussed this view and advanced a new one, according to which the weight differences between litters of different sizes are due to the limitations of the mother in assimilating and in providing nourishment for the young   This view is well supported by our observations (to be reported in a subsequent paper) that the growth rate of the suckling young depends upon the litter size in the same manner as does birth weight

The relationship between litter size and litter weight is not explained by either theory (1) equipartition of a limited amount of a hormone which induces parturition or (2) equipartition of a limited amount of nutrition provided by the assimilating capacity of the mother   Our experiments on the growth rate of suckling young in the litters of various sizes strongly favor the second idea as a partial explanation

The present results show that although birth weight depends on a variety of factors the litter size is (within the same strain) the most important   Since

$$\Delta W / W = k(\Delta N / N)$$

we have to suppose that the average increment of litter weight resulting from a unit increase in N is directly proportional to W and inversely proportional to N This signifies a proportionality between N and the nutritive drain upon the mother, as well as an equipartition among the members of the litter   The remarkable fact that

K is practically identical, within very narrow limits (Figs 1, 2, 3), for various mammals, shows that the partition coefficient is non-specific In the case of multiparous mammals, then, we have the possibility of a direct test of the theory of the partition of materials in "heterogonic" growth, of which use has been made by Robb (1929) and Teissier (1934)

<div align="center">IV</div>

<div align="center">SUMMARY</div>

In multiparous mammals there is a definite relation between the litter size N and the total weight of the litter W Reasons are given showing that this relationship is independent of the mechanism of parturition

For various forms $W = N^{-k} + const$ Hence the average increment of W due to unit increase of N is directly proportional to W, inversely to N This signifies proportionality between N and nutritive drain upon the mother, as well as equipartition among the members of the litter K is non-specific, and is therefore regarded as a partition index

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ON THE MODIFICATION OF TEMPERATURE
CHARACTERISTICS I

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I

Attempts to modify experimentally the curves relating frequencies or velocities of vital processes to temperature give evidence concerning the nature of temperature characteristics (Crozier, 1924–25) In general these frequencies (F) or speeds are describable in terms of the equation $F = k \, exp \, (- \, \mu/RT)$, where μ is the "temperature characteristic" The empirically determined magnitudes of μ are distributed in such a way as to indicate (Crozier, 1925–26 b, Stier, 1932–33) that they exhibit specific significance This is consistent with their unitary behavior in inheritance in a tested case (Pincus, 1930–31), and with their association with particular activities in a given individual organism (cf Crozier and Stier, 1926–27 b, Stier and Wolf, 1932–33, Crozier, Tang, and French, 1933–34, French, Kohn, and Tang, 1934–35, Crozier, Pincus, and Renshaw, 1934–35) It is consequently important to examine the modifiability of μ in a number of cases Two general possibilities are open either the frequency F may be changed by experimental treatments, without altering μ, or μ may be changed In the latter event two results are possible since homologous vital processes tend to show one of several discrete magnitudes of μ, the alteration may be by an abrupt step from one to another of these, or μ might be found changed gradually and in an indeterminate way In the event that the last possibility is realized, no evidence concerning the initial significance of μ is obtained one way or the other, and the situation must be further analyzed But in the other cases important evidence is had that the interpretation of μ as the molecular energy of activity of a specific chemical process (Crozier, 1924) may be called for

The situation is complicated by the fact that definite critical temperatures occur (Crozier, 1925-26 a) on either side of which distinct values of μ obtain for a given activity In cases of this sort we may expect to find, accepting the theory of specific significance of μ, that by experimental treatment one or the other value of μ may be made to prevail over a greater range of temperatures, with consequent shift of the critical point   Essentially this effect has been realized in experiments with preparations in progressive deterioration (Crozier and Stier, 1924-25, 1926-27 a)   Experiments with the frequency of heart beat in *Dendronotus* under the action of alkaloids (Crozier and Stier, to be published shortly) also demonstrate this (Crozier, Stier, and Pincus, 1929)

Indications are not wanting in the literature that treatment with a drug may modify the temperature coefficient of a given activity, but these require to be controlled by the demonstration that during the observations the activity in question has not also been altering as a function of time

II

The isolated cloacal end of pedate holothurians such as *Mulleria* (*Stichopus*), *Holothuria*, *Thyone*, exhibits, in sea water, rhythmic pulsation of the cloaca and terminal sphincter for many hours (Crozier, 1915, 1916)   From measurements of the frequency of pulsation in *Mulleria* (*Stichopus*), $\mu = 12,200$, in *Holothuria captiva*, $\mu = 20,500$, in *Thyone*, 8,500, but in the intact *Thyone*, $\mu = 12,200$ (Crozier and Stier, to be published)   These magnitudes appear to be of the group (including 11,300, 16,200) found for respiratory movements in general (cf Crozier, 1925-26 a, Crozier and Stier, 1924-25)

Experiments with *Holothuria tubulosa* of the Mediterranean, at Banyuls, gave for frequency of pulsation of the isolated cloacal end (cloacal sphincter) data collected in Fig 1   Between 10 and 20°, $\mu = 20,400$ cal , from 20 to 30°, $\mu = 8,000$ Above 20° the latitude of variation in frequency is markedly less than below   Within each range, however, the relative variation is independent of temperature (cf Crozier and Federighi, 1924-25 b, Crozier, 1929, Stier, 1932-33)   With the isolated cloacal ends deterioration is of course more rapid at the higher temperatures, and one finds (as shown in Fig 1) that in

different cases the maximum frequency of pulsation occurs at 28 5° or at 25°, but never beyond 30°, a decrease of frequency beyond the maximum was found to entail incomplete recovery on return to lower temperatures (omitted from Fig 1), whereas, over the periods considered, the effect of altering the temperature was otherwise found to be com-
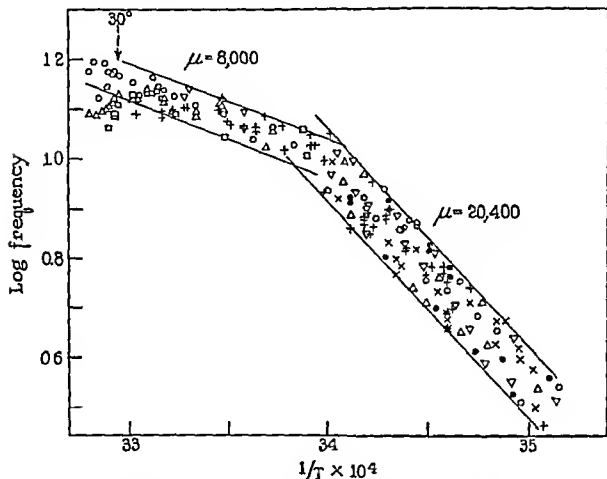
FIG 1 Relation between frequency of pulsation in amputated cloacal ends of *Holothuria tubulosa* and temperature in sea water The plotted points are averages of five to ten readings of time for one complete cycle The readings for one individual (△) have been multiplied by 1 34 for another (▽) by 1 22 to bring them into the field of the others Readings in some cases made with rising temperatures in others with falling Constant rates of pulsation in sea water at constant temperature for 36 to 48 hours

pletely reversible The occurrence of maximum frequency between 20 and 30° is determined by the age of the preparation and by the length of the isolated cloaca, incomplete cloacas degenerate sooner The upper critical temperature (30°) is well above that normal for *H tubulosa* at Banyuls, the lower limit for sustained pulsation was 10°, all movement

It is to be noted that the value of μ with increased NaCl agrees quantitatively with that assigned to the upper segment of the tempera ture curve in sea water (Fig 1)

The outcome of the experiment thus agrees with the results of other tests, in which amputation of parts has induced change of μ, but change to a new, definite value, rather than alteration by slow progressive steps (Crozier and Stier, 1924–25, 1926–27 *a*, Wolf, 1927–28)
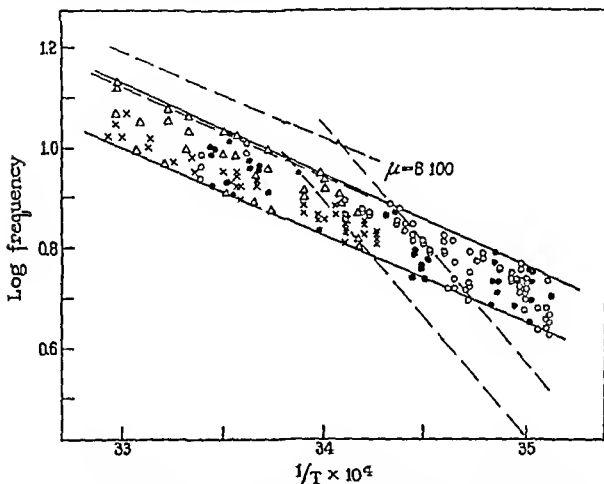
FIG 2 In sea water with NaCl content increased, without increase of osmotic pressure, the temperature curve is altered Lines added from Fig 1 For one individual the readings have been multiplied by 1 38

IV

The effect of increasing the NaCl content of the sea water has been to alter the temperature characteristic of the frequency of pulsation in amputated cloacal ends of *H tubulosa* In doing this, the frequency at higher temperatures has been lowered, at lower temperatures in creased, while within a certain range of temperature the frequency

trolling position It may be suggested as an important deduction that inquiries of the sort here initiated may provide a new and necessary method for the study of the effects of salts, and indicate a basis for the reconciliation of otherwise contradictory findings If the primary effect consists in a change of temperature characteristic it may be comparatively irrelevant whether a given activity is enhanced or diminished This consideration applies with equal force to the treatment of the effects of alkaloids

<p style="text-align:center">V</p>

SUMMARY

The temperature characteristics for frequency of pulsation in isolated cloacal ends of *Holothuria tubulosa* are $\mu = 20,400$ from 10 to 20°, 8,000 from 20 to 30° The critical temperatures 10°, 20°, 30° are related to thermal conditions in the environment

In sea water with doubled NaCl content $\mu = 8,000$ from 10 to 30° This is consistent with the specific nature of the temperature characteristics

The primary effect of increased NaCl is to alter the temperature characteristic curve At low temperatures the frequency is increased, at higher temperatures diminished The effect upon frequency is thus of less significance It is pointed out that such relationships must he taken into account in analyzing the action of reagents upon organisms

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STUDIES ON BLOOD COAGULATION

III On the Constancy of the Hydrogen Ion Concentration during the Coagulation of Fibrinogen by Thrombin

By HARRY EAGLE* and J P BAUMBERGER

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Kugelmass and Stuber and Lang have reported that the coagulation of blood or solutions of fibrinogen is accompanied by an increased alkalinity of the free fluid, which the former definitely ascribes to an absorption of hydrogen ions by the clot Hirsch, however, reports an increased acidity, and suggests that it is the actual cause of coagulation, and Hekma in claiming that fibrinogen is the alkaline hydrosol of fibrin, implies that he believes its coagulation to be due to an acidification of shed blood Finally, Havard and Kerridge report that the time-pH curve of shed blood is unaffected by coagulation

Clearly, the matter requires reinvestigation If it could be proved that the transformation of fibrinogen to fibrin is invariably accompanied by a change in the hydrogen ion concentration of the free fluid, such a change would offer a valuable clue to the essential difference between the two proteins

With this in mind, thrombin was added to solutions of horse and rabbit fibrinogen, and the pH followed potentiometrically

Fibrinogen and thrombin were prepared as described in a preceding paper (Eagle) Error due to loss of CO_2 was minimized by using a fibrinogen solution in equilibrium with air The prothrombin used in preparing the thrombin was adjusted to approximately pH 6 5 prior to the addition of $CaCl_2$, in several experiments using dilute NaOH

the phenomenon of coagulation, the change in the pH of the unbuffered fibrinogen solutions used in the foregoing experiment would have been of the order of 1–2 full pH units Even if only one equivalent of free

TABLE I

The Constancy of pH during the Coagulation of Fibrinogen by Thrombin

		Observed potential	Maximum shift in pH
		mr	
Phosphate buffer at 6 64		0.2834	
Fibrinogen solution (1 34 per cent in 0 85 per cent NaCl)		0.2870	
Temperature 20°			
p m			
3 19	0 5 cc thrombin containing 1/400 м phosphate buffer at pH 6 64 added to 10 cc fibrinogen solution		
3 20		0 2875	
3 22		0 2875	
3 24		0 2894	±0 03*
3 25	(Clot)	0 2894	
3 29		0 2894	
3 31½	1 5 cc unbuffered thrombin added to 10 cc fibrinogen		
3 32½		0 2891	
3 33½		0 2891	
3 35	(Clot)	0 2891	0
3 36		0 2891	
3 41		0 2891	
3 48	Supernatant obtained by expressing the clot	0 2890	
3 52½	0 3 cc unbuffered thrombin added to 10 cc fibrinogen		
3 53		0 2874	
3 56		0 2882	
3 59		0 2879	±0 013
4 00	(Clot)	0 2879	
4 18		0 2879	
4 35	Supernatant expressed from clot contains 0 11 per cent protein therefore more than 90 per cent of the fibrinogen had coagulated		

* Within limit of error of the method used

H or OH were liberated per mole fibrinogen, this would mean 0 013 m -eq per gm fibrinogen,[1] and approximately 0 00017 m eq per cc

[1] Assuming a molecular weight for fibrinogen of 75 000, intermediate between the accepted values for serum albumin and serum globulin

STUDIES ON BLOOD COAGULATION

IV The Nature of the Clotting Deficiency in Hemophilia

By HARRY EAGLE

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Numerous explanations, recently summarized by Wohlisch, have been offered for the delayed coagulation observed in hemophilia, the presumable cause of the bleeding tendency According to Sahli, Nolf, Morawitz, Minot and Lee, Howell and Cekada, and Christie, the platelets of hemophilic blood are excessively stable, failing to dis integrate and yield the platelet factor essential for coagulation How ever, Addis Klinger, and more recently, Feissly and Fried maintain that the platelets are functionally normal The latter find hemo philic blood to be deficient in prothrombin, this deficiency presumably explaining the retarded coagulation Addis, however, reports a *qualitative* difference in so far as the prothrombin of hemophilic blood, although normal in quantity, is more slowly activated by Ca and the cell factor to form thrombin These findings are disputed by Howell and Cekada

Clearly, all these theories cannot be correct, and the experiments to be described seem to confirm the observations of Addis The plate lets of normal and hemophilic[1] blood compared quantitatively with respect to their effect upon the coagulation of platelet-free horse or human plasma, are found to be indistinguishable Both types of platelet suspension accelerate coagulation to the same degree, and both accelerate the production of thrombin from prothrombin to the same degree, no matter what type of plasma is used, whether oxalated,

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[1] Dr W B Chew of the Boston City Hospital Thorndyke Laboratory kindly furnished the blood from hemophilic patients under treatment or observation for use in these experiments

incubated for $\frac{1}{2}$ hour, and its thrombin activity then tested by adding serial quantities to 1 cc of pure horse fibrinogen solution   There was no demonstrable difference between any of the four hemophilic plasmas and five normal human controls

2 *The Coagulating Activity of Hemophilic Platelets Is Normal* — Serially decreasing quantities of similar washed suspensions of normal and hemophilic platelets were added to 1 cc of citrated (*a*) normal human plasma, (*b*) hemophilic plasma, (*c*) horse plasma, and (*d*) horse prothrombin solution, CaCl₂ was then added and the coagulation time

TABLE I

Showing That There Is no Difference in the Coagulating Activity of Normal and Hemophilic Platelets

(Numbers in the body of the table indicate coagulation time in minutes)

	Type of platelets	Platelet suspension				
		0 4 cc.	0 1 cc	0 025 cc	0 0062 cc.	0 cc.
Normal human plasma	Normal	4	6	9	15	18
oxalated	Hemophilic	4½	6	10	17	19
Normal human plasma	Normal	5	7	9	12	25
citrated	Hemophilic	5	6	8	12	25
Hemophilic plasma ci	Normal	9½	14	25	40	90
trated	Hemophilic	8½	13	19	32	90
Hemophilic plasma ci	Normal	16	23	35	60	180
trated	Hemophilic	17	28	40	65	185
Horse plasma oxalated	Normal	10	20	42	No clot in 2 hrs	
	Hemophilic	6	19	40	No clot in 2 hrs	

noted   The human plasmas had been freed of platelets by centrifugation, the horse plasma by centrifugation and Berkefeld filtration The amount of 0 1 м CaCl₂ used was usually 1/5 the plasma volume for human citrated plasma (0 5 per cent), and 1/2 the plasma volume for horse plasma   With oxalated plasmas, four times the amount necessary to combine with the oxalate stoichiometrically was used   In each experiment, the total value was always brought to two times the volume of plasma by adding 0 85 per cent NaCl   In no case was there any significant difference in the coagulating activity of normal and hemophilic platelets (Table I)

3 *The Prothrombin Content of Hemophilic Plasma Is Normal* —

Prothrombin was prepared from platelet free normal and platelet-free hemophilic plasma   To 1 cc of each prothrombin solution was added 0 1 cc of CaCl₂ and the course of thrombin production followed quantitatively (*cf* Eagle)   As is shown in Fig 1, the hemophilic prothrombin is converted to thrombin much more slowly than is normal prothrombin, but the amount of thrombin ultimately formed is normal   Since the latter depends solely upon the prothrombin
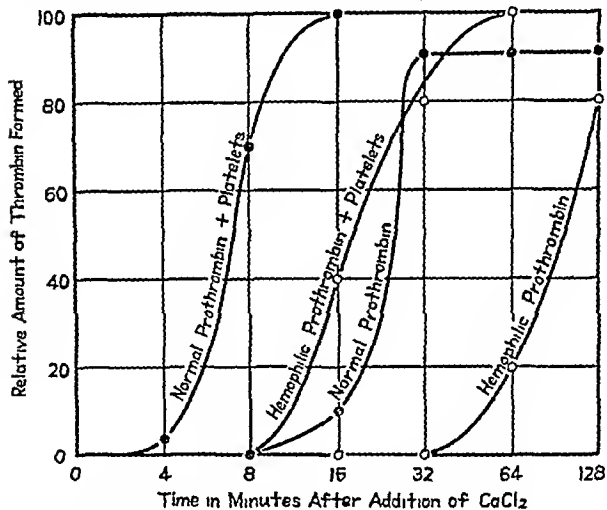
Fig 1 Showing the slow formation of thrombin from hemophilic prothrombin as compared with normal prothrombin

concentration (Eagle), it follows that prothrombin of the hemophilic plasma is normal in quantity, but is converted to thrombin only with difficulty   The addition of a fixed quantity of platelets accelerates the thrombin production in both solutions markedly, but the hemophilic prothrombin still lags behind the normal prothrombin   However, the amount of thrombin ultimately formed is not significantly affected by the platelets

5 *The Effect of Cephalin upon Hemophilic Blood.*—One additional point may be briefly mentioned   In the case of normal plasma, platelets and cephalin are to a certain extent interchangeable   both accelerate the transformation of prothrombin to thrombin, and thus shorten the coagulation time (Table)   As has just been seen, the retarded coagulation of hemophilic blood is largely due to the retarded formation of thrombin   This can be compensated for by adding an excess of platelets   Cephalin, however, is unaccountably ineffective in accelerating thrombin formation and coagulation in hemophilic blood

TABLE III

Show that Cephalin, Unlike Platelets, Does not Markedly Affect the Coagulation of Hemophilic Plasma

(0.1 cc of plasma plus varying quantities of cephalin plus 0.2 cc 0.1 M CaCl₂ plus salt solution to a total volume of 1 cc   Figures in the body of the table represent coagulation time in minutes.)

	Cephalin							
	0.1	0.1	0.05	0.01	0.001	0.005	0.0001	0
Normal human plasma*	15	12	13	17				32
Normal human plasma	54	14	64					9
Horse plasma†	15	13	12	14	16	25	35	>180
Hemophilic plasma	120	150	150	210				210
Hemophilic plasma	>120	>120	>120	>120				>120
Hemophilic plasma	25	16	15					30
Hemophilic plasma	>60	25	35					40

* Platelets not removed
† Berkefeld filtrate

(Table III)   This puzzling fact, first noted by Mills, requires further investigation, and may provide a clue to the nature of the missing factor in hemophilic plasma

SUMMARY

Despite their reported stability, the platelets of hemophilic blood function normally   The prothrombin content of such plasma is also normal   Confirming the findings of Addis, the delayed coagulation observed in hemophilic blood is due to an unexplained retarded activation of prothrombin to thrombin   The addition of an excess of plate-

lets, whether normal, hemophilic, or animal in origin, accelerates thrombin production and makes hemophilic blood clot normally, but cephalin, despite the fact that it accelerates thrombin production in normal plasma, is unexplainedly ineffective when added to hemophilic plasma

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THE ELECTRIC IMPEDANCE OF HEMOLYZED SUSPEN-
SIONS OF MAMMALIAN ERYTHROCYTES

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Determinations of the complex impedance (measured in terms of resistance and capacitance) have already provided a means of characterizing the surface of the red corpuscle (1)   This paper is concerned with an extension of this method to a study of hemolysis

EXPERIMENTAL PROCEDURE

The impedance of a suspension of cells may be represented as a resistance and a parallel capacitance which, per centimeter cube of suspension, are referred to as the resistivity R (ohms cm) and the capacity C (micromicrofarads-cm $^{-1}$) of the suspension

The values of R and C at frequencies between 1/4 and 2000 kilocycles per second were measured with a Wheatstone bridge using a substitution method (1)   The electrolytic cell containing the corpuscles is replaced by a similar cell filled with a salt solution of the same resistance and connected in parallel to a condenser (of negligible inductance)   The function of the bridge is solely to indicate the existence of electrical equivalence in the two cases   The capacitance of the suspension is obtained by adding the static capacitance of the comparison cell to the capacitance of the parallel condenser   The static capacitance of the comparison cell equals

$$\frac{D}{4\pi K} \quad \frac{9}{10} = \frac{7\,1}{K}\ \mu\mu F$$

where D is the dielectric constant of water (79 at the experimental temperature of 21 4 C), and K is the cell constant

The same procedure of substitution was used from 2000 to 16 000 kilocycles/sec but a resonance method was used instead of the bridge to indicate the existence of equivalence

The electrolytic cells are cylindrical with one electrode mounted on a micrometer screw which allows a fine adjustment of the resistance of the comparison cell to be made   The electrodes are of platinum and coated with platinum black to

decrease the polarization. At the lowest frequencies, this polarization at the electrodes may be appreciable. Its influence is eliminated by making measurements at two different distances of the electrodes. The influence of the polarization is the smaller the further the electrodes are apart. In certain cases, an electrolytic cell was used where the maximum electrode separation was 20 cm. The area was 10 sq cm and the volume about 300 cc.

Normal Corpuscles

At low frequencies the resistivity of the red corpuscle is very high compared to that of the serum,[1] and measurements of the complex impedance indicate that this high resistivity is derived from the surface of the corpuscle, while its interior is composed of a fluid having a resistivity and a dielectric constant not greatly different from that of normal serum.[2] The surface of the corpuscle acts as an electric condenser with a rather small power loss.

The values of C and R, for blood of the rabbit, are shown in Fig 1 as functions of the frequency. At the lower frequencies C and R are also shown in a magnified scale. Our earlier measurements (4) did not go below 1 kilocycles and from these it appeared that C and R were constant at the lower frequencies. The present extension of the frequency range down to $\frac{1}{2}$ kilocycle shows, however, that this is not the case, but that a rise in C and R occurs at the lowest frequencies.

A unit surface of the corpuscle may be represented as a complex impedance, the parallel components of which are the surface capacity C_n (micromicrofarads/cm^2) and the surface resistivity R_n (ohms/cm^2).

The phase angle θ, which is always small, is given by $\tan \theta = \dfrac{1}{2\pi n C_n R_n}$

where n is the frequency of the alternating current

[1] This may be concluded from the excellent agreement between the observed value of the resistance of a suspension of red corpuscles, and that calculated theoretically (2) assuming infinite resistivity of the corpuscles. More direct evidence is obtained by measuring the resistance of a dense mass of cells, packed by centrifugation. Values of the resistance from 30 to 40 times the resistance of the serum are then obtained. The temperature coefficient of this resistance (measured between 0 and 37°C) is found to be the same as that of the serum, which suggests that to a great extent this resistance is due to the serum still left between the corpuscles

[2] The resistivity of the interior fluid is about twice that of the serum (3) (rabbit, sheep, and chicken)

From the observed values of C and R, C_m and R_m may theoretically be derived if we know the resistivities and dielectric constants of the inter and intracellular fluids, and the form and volume concentration of the corpuscles A discussion of this problem will be given at another place For the spherical form, a theory has been worked out by
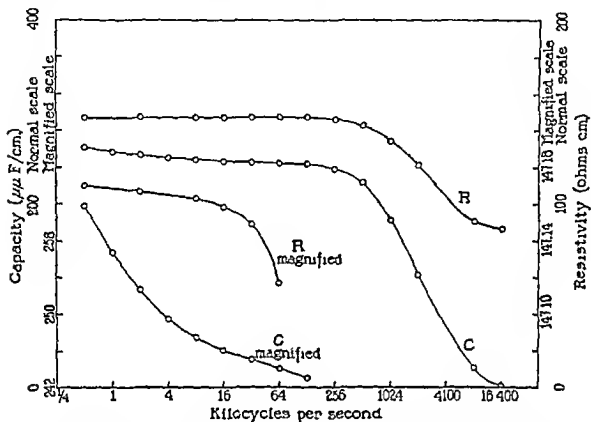
FIG 1 Resistivity (R) and capacity (C) for a suspension of normal rabbit corpuscles

Cole(5) At sufficiently low frequencies the following formulae are valid

$$C_m = \frac{2/3\,(C - C)\,(1 + \tfrac{1}{2}\rho)^2}{\alpha\,a\,\rho} \qquad (1)$$

$$\frac{1}{R_m} = \frac{2/3\,(1/R - 1/R)\,(1 + \tfrac{1}{2}\rho)^2}{\alpha\,a\,\rho} \qquad (2)$$

where $2a$ is the major axis of the corpuscle, α a constant dependent on the form of the corpuscle, and ρ the volume concentration The value of α is not known, but we have carried through the calculation by assuming the corpuscle to be equivalent to a sphere, in which case

size of the corpuscles, since by (1) and (2) such values are given by $(C - C_s)$ and $(1/R - 1/R_s)$, respectively Curves in which these values are plotted accordingly afford the most practicable way in which the present method can be used to characterize the corpuscle surface Since C_s is small and constant, these relative values of C_m are practically given by the values of C At the higher frequencies the calculation of even relative values involves the form of the corpuscle Owing to the peculiarity of the form, an exact theory would be difficult, but
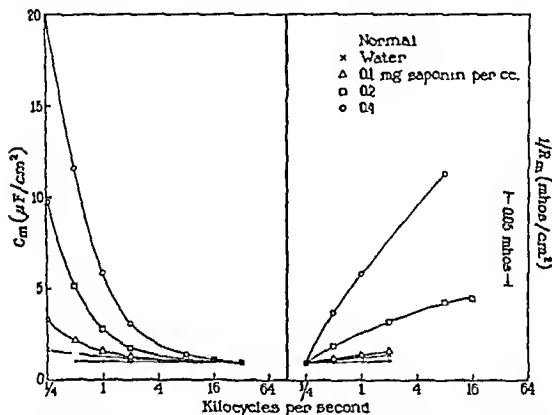
FIG 2 Surface conductivity $(1/R_m)$ and surface capacity (C_m) for normal and hemolyzed rabbit corpuscles

calculations have been carried out under simplifying assumptions as to the form These show that C_m and R_m remain constant or very nearly so at high frequencies The value of θ is small at all frequencies

For a tentative interpretation of the surface impedance of the red corpuscle, it may be assumed that the latter is surrounded by a membrane which has a small selective ionic permeability When placed in an electric field, it therefore shows a small conductivity, due to the small ionic transport across it, and a polarization due to the difference in permeability to anions and cations At high frequencies, the in-

fluence of this polarization is negligible,[3] and the value of C_m is then the static capacitance of the membrane, and $1/R_m$ represents the conductivity due to the ionic movement of the ions in the membrane, as well as that due to any dielectric loss present. Since dielectric constants generally depend on the frequency, and since a dielectric loss is probably present, neither C_m nor R_m could be expected to remain constant at high frequencies. However, a more correct method of calculating C_m and R_m is required before the expected decrease can be established. At low frequencies the influence of the polarization becomes appreciable, leading to an increase of C_m and R_m, as observed.[4]

Hemolysis

General. —In applying these methods to a study of hemolysis, we have used suspensions of red corpuscles hemolyzed with water, with various chemical lysins (including saponin, complement and amboceptor, sodium taurocholate, and digitonin) and by freezing and thawing. Rabbit corpuscles were used throughout except in the experiments with complement and amboceptor, which were carried out with sheep corpuscles. Measurements have been made on completely hemolyzed suspensions only. It has not been found possible to obtain significant results for partially hemolyzed suspensions, owing to a marked dependence of the values of C and R on stirring.

In Fig. 3 are shown the curves for C and R obtained for a suspension of rabbit corpuscles hemolyzed by adding three parts of water to one

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[3] Compare with observations on polarization at metal electrodes (7)

[4] (Added February, 1935) Recent work has shown that there may be another explanation of the rise of C and R at low frequencies. Since the red corpuscle migrates in an electric field, it must be charged and therefore must have a diffuse ionic double layer at its surface. Some small part of the conductance of a cell suspension must be derived from this double layer, this being what is generally referred to as surface conductance. On theoretical grounds we should expect the electric current passing through the double layer to become polarized and this polarization would result in an increase of C and R as the frequency decreases. Such increases have actually been observed in suspensions of non conducting particles such as rouge, sulfur, and paraffin oil. While the increase in C and R, at low frequencies, may be of this origin in the case of normal corpuscles, in the meantime it still seems to us reasonable to suppose that the still greater increase of C and R observed in the case of hemolyzed corpuscles, is due to an increased permeability of the cell membrane, as stated in the text.

part of packed corpuscles The large value of C and the general simi larity of these curves to those for normal corpuscles give evidence of the presence of "cells" in the hemolyzed suspension A difference from normal corpuscles is shown by the more pronounced rise of R and C at the lower frequencies

For the theoretical interpretation, the determination of the re sistivity of the intercellular fluid of the hemolyzed suspension is re quired The intercellular fluid cannot be separated by centrifugation,
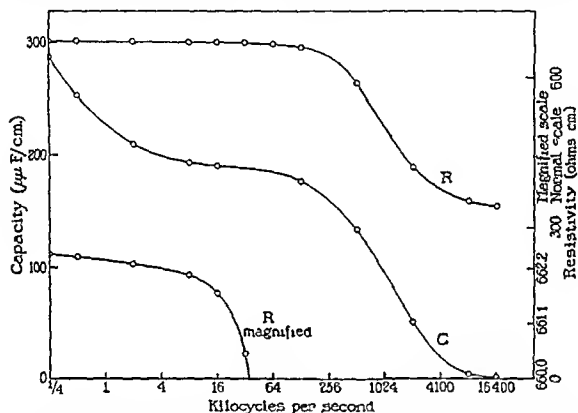
FIG 3 Resistivity (R) and capacity (C) for a suspension of rabbit corpuscles hemolyzed by adding three parts of water to one part of packed corpuscles

but the following two indirect methods have been used for the determination

1 At the frequency of 16,000 kilocycles the resistivity of the hemo lyzed suspension becomes nearly constant (Fig 3) By an extrapola tion the resistivity at "infinite frequency" is determined At infinite frequency, the impedance of the cell membranes is negligible and there fore this extrapolated value, R_∞, is the resistivity which would be obtained at low frequency if all the cell membranes could be removed without any other change taking place If we make the reasonable

assumption that after hemolysis the intercellular and intracellular fluids are identical, it follows that R_∞ gives their resistivity

2 The addition of sufficient saponin to the suspension completely destroys the membranes of the cells, leaving a homogeneous fluid, as will be shown later   With a suitable correction for the influence of the added saponin, the resistivity, R_{sap}, of this fluid should equal that of the intercellular and intracellular fluids
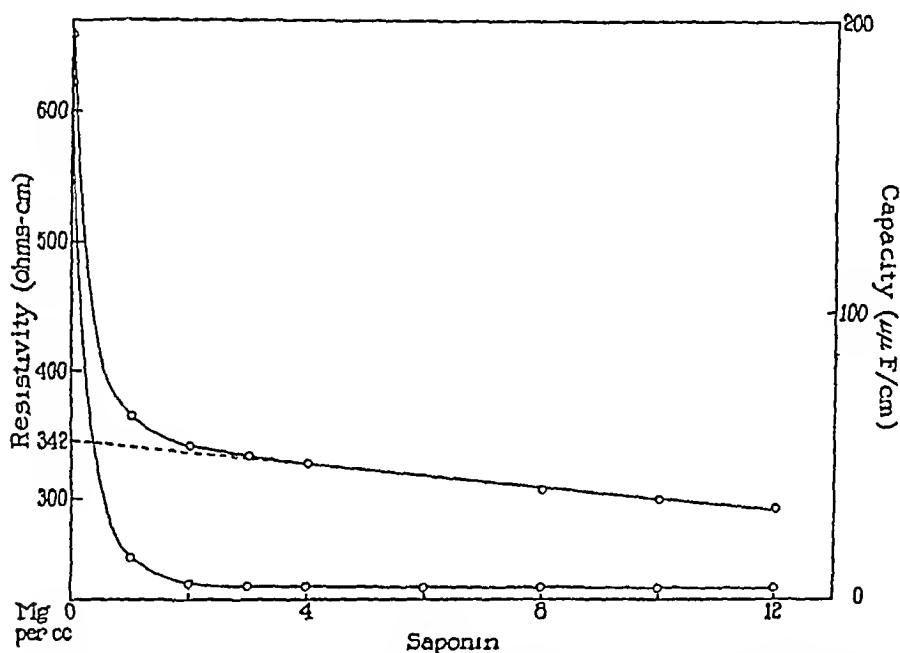
FIG 4 Stromatolysis of the hemolyzed suspension of Fig 3 with saponin, in order to obtain the resistivity of its suspending fluid   Resistivity (R) and capacity (C) as functions of saponin concentration

The values obtained by these two methods are found to be in satisfactory agreement   The average value is used as the resistivity of the intercellular fluid

The procedure may be illustrated by the results on the suspension to which Fig 3 refers   For R_∞, the value $R = 340$ ohms is obtained In Fig 4 are shown C and R, at 128 kilocycles, as functions of the amount of saponin added (given in milligrams per cubic centimeter of suspension)   The destruction of the cell membranes is indicated by the decrease of C and R When more than 4 mg of saponin have been added, R changes slowly and at a constant rate, and C is very

nearly zero   At the same time, it is found that R and C become independent of the frequency   (The small final value of C of about $7 \mu\mu F$ corresponds to a dielectric constant nearly equal to that for water)   These facts indicate that complete destruction of the membranes has taken place   Extrapolating the straight part of the curve for R back to zero concentration of saponin, the value $R_{\text{sap}} = 342$ ohms is obtained   This value is in good agreement with the value for $R_\infty = 340$ ohms   The average $R_1 = 341$ ohms is taken as the value for the resistivity of the intercellular fluid

We now make use of the following formula (2),

$$\frac{\frac{R_1}{R} - 1}{\frac{R_1}{R} + \lambda} = \rho \, \frac{\frac{R_1}{R_2} - 1}{\frac{R_1}{R_2} + X} \tag{4}$$

where R, R_1, and R_2 are the resistivities of the suspension, suspending fluid, and suspended cells respectively, where X depends on R_2/R_1 and on the form of the cells, and where ρ is the volume concentration   The form of the hemolyzed cells is unknown, but there is evidence that they change to the spherical form just before hemolysis occurs (6), and although we do not know that they retain this form, we have assumed them to do so, giving $X = 2$ The error introduced, even with a considerable deviation from the spherical form, is comparatively small

We introduce now

$$\rho_o = 2 \rho \, \frac{1 - \frac{R_1}{R_2}}{\frac{R_1}{R_2} + 2} \tag{5}$$

where R_2 is the resistivity of the hemolyzed cell at low frequencies   We term ρ_o the equivalent non conducting volume concentration   It is the true value of the volume concentration only if $R_2 = \infty$, that is, if the hemolyzed corpuscles are non-conducting at low frequencies   The value of ρ is determined from

$$\rho = 2 \, \frac{1 - \frac{R_1}{R_o}}{\frac{R_1}{R_o} + 2} \tag{6}$$

which is obtained by substituting (5) in (4) with $X = 2$ and where R_o is the low frequency and practically constant resistivity of the suspension

The value of ρ_o is most conveniently expressed as a fractional part of ρ_1, where ρ_1 is the volume concentration of the original suspension As an example of the calculation of ρ_o/ρ_1, consider again the suspension of Fig 3 The low frequency resistivity of the suspension is $R_o = 662$ ohms The resistivity of the suspending fluid was determined above to be $R_1 = 341$ ohms Therefore, from (6), $\rho_o = 38\ 5$ per cent The packed suspension, from which the hemolyzed suspension was prepared, was found, from its electrical resistance, to have a volume concentration of 95 per cent The addition of water in the relation 1 3 reduces this volume concentration to $\rho_1 = 23\ 8$ per cent, giving $\rho_o/\rho_1 = 1\ 61$

Water —Several experiments were carried out in which the volumes of water added to produce lysis were varied from three to nineteen parts of water for each part of packed corpuscles The values of C and R showed no change with time, during observations extending over several hours No measurements could be made, however, until a few minutes after hemolysis was complete Values of ρ_o/ρ_1 between 1 45 and 1 65 were obtained, with no apparent dependence on the volume of water used The average was $\rho_o/\rho_1 = 1\ 54$ This factor gives a reasonable value for the swelling which the corpuscle would undergo before hemolysis takes place This agreement shows that the resistivity (at low frequencies) of the hemolyzed corpuscles is high as compared with that of the suspending fluid Since we do not know either the exact volume of the hemolyzed corpuscles or their form, an accurate determination of the resistivity of the hemolyzed corpuscles is not possible by this method

The calculation of the surface resistivity R_m and the surface capacity C_m of the hemolyzed corpuscles is carried out by the same method as used for normal corpuscles For this purpose we assume that the hemolyzed corpuscle is spherical, giving $\alpha = 1\ 50$, and that its volume is ρ_o/ρ_1 times that of the normal corpuscle, which for rabbit is taken to be 57 μ^3 (6) From the volume, the diameter is determined The dielectric constant of the inter- and intracellular fluids is taken as equal to that of water, $D = 79$

In Fig 2 C_m and $1/R_m$ are given as functions of the frequency
The values show no systematic dependence on the amount of water
used to produce lysis, and the curves represent averages of all experi
ments   Since the values of $1/R_m$ can be expressed only as differences
from an undetermined constant, the curves for $1/R_m$, for water, as
well as for the other lysins used, have arbitrarily been made to agree
with the one for normal corpuscles, at 250 cycles/sec.   The constant
value of C_m, obtained at high frequencies, is the same as that found
for normal corpuscles, which shows that lysis produces no change in
the static capacitance of the membrane   The injury to the corpuscles
due to the lysis is shown by the changes in C_m and $1/R_m$, at low fre
quencies   These changes are presumably due to an increased ionic
permeability of the membrane, and it may be worth noting that since
the lysis produces a change in the frequency dependence of C_m and
$1/R_m$, this increased permeability cannot be due to a rupture of the
membrane, as has sometimes been proposed, but must be due to an
actual change in the membrane itself

Saponin —An important difference between osmotic lysis and
chemical lysis is shown by the fact that in the latter case, C and R
do not become constant until a considerable time after hemolysis is
complete, during which period both decrease   This may be seen from
Fig 5 which shows C and R, measured at 128 kilocycles, plotted
against the time, after the addition of 0 1 mg/cc of saponin to a
39 per cent suspension of rabbit corpuscles in saline   The initial
increase of R is due to the release of the hemoglobin and gives a graphi
cal representation of the course of the lysis   This is complete in 8
minutes C and R do not become constant until 30 minutes later
As the concentration of saponin is increased, these final values of C
and R decrease until, with the addition of sufficient saponin (compare
Fig 4), R reaches a final low value, while C reaches the low value
(about 7 $\mu\mu F$) characteristic of a homogeneous aqueous solution
At the same time R (as well as C) becomes independent of the fre
quency   The curves in Fig 6 represent these final values of C and R,
as functions of the frequency, for suspensions of rabbit corpuscles in
saline, hemolyzed with three different quantities of saponin   The
original volume concentration of the suspension was in each case 39
per cent, and in a suspension of this concentration 0 02 mg of saponin
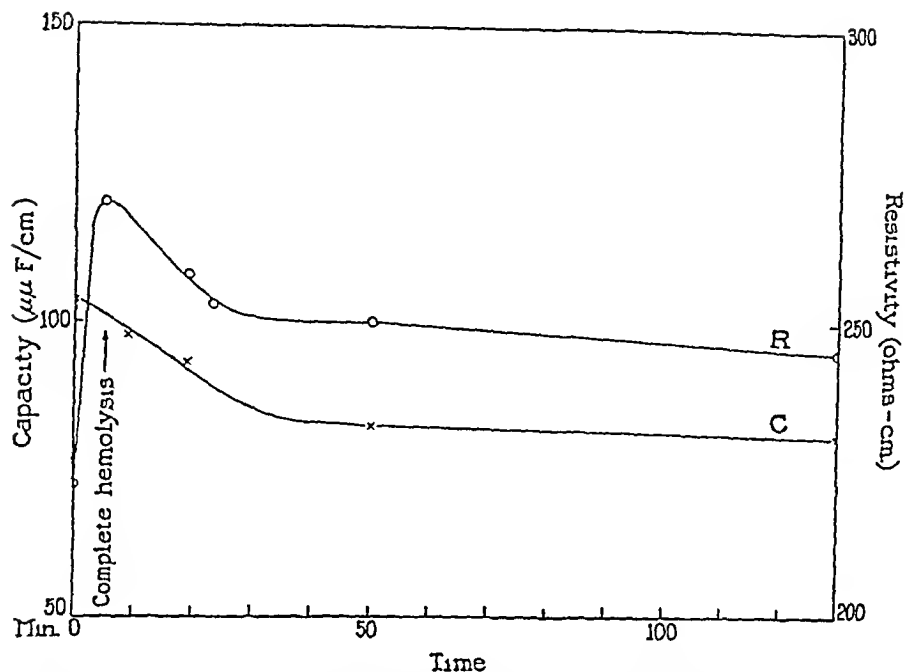
FIG 5 Resistivity (R) and capacity (C) as functions of time after the addition of 0 1 mg /cc of saponin to a 39 per cent suspension of normal rabbit corpuscles in saline
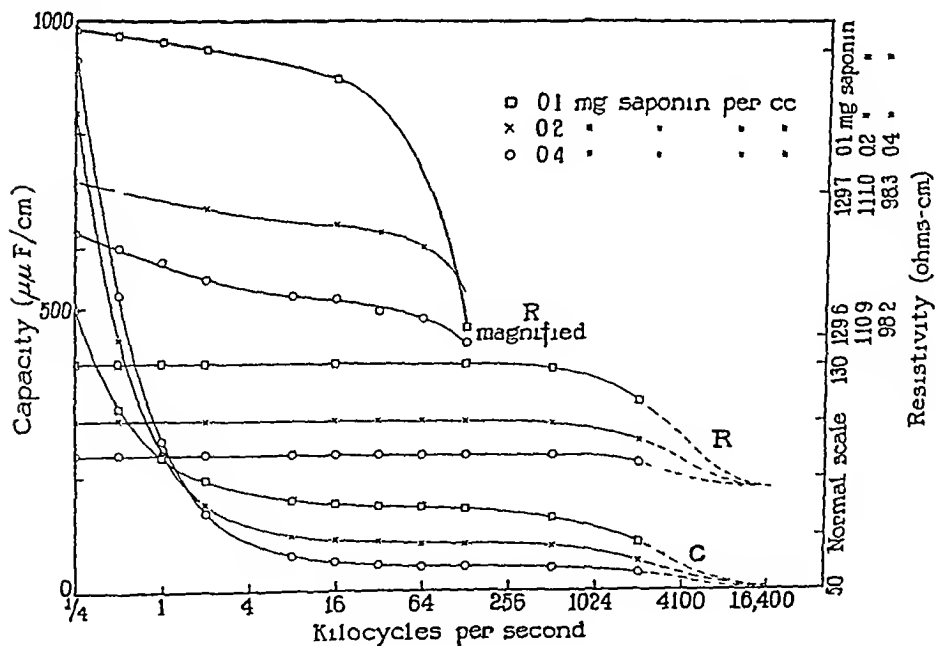
FIG 6 Resistivity (R) and capacity (C) for suspensions of rabbit corpuscles hemolyzed by the addition of saponin

per ee of suspension is required to produce 50 per cent hemolysis
The form of the curves, at low frequencies, shows evidence of injury to
the membranes of the corpuscles of the same type as that found after
osmotic lysis, and to an increasing extent as the concentration of the
saponin increases

From the facts given it follows that with a large amount of saponin
the membranes of all the corpuscles become completely permeable, the
reasonable inference being that disintegration has taken place   For
the case of the suspensions to which saponin has been added in more
moderate concentrations, it is found most satisfactory to assume that
the decrease of C and R, which takes place after lysis, is due to the
disintegration of the membranes of a certain number of the corpuscles
while the remainder are injured to a slight extent only, retaining in
particular a high resistance, as compared with that of the intercellular
fluid   These remaining cells, as regards our measurements, are the
only "effective" cells of the suspension   As the concentration of
saponin is increased, the number of disintegrated corpuscles increases,
until with sufficient saponin, all corpuscles are so affected   The es
sential point which is brought out by this evidence is that as the injury
proceeds, the permeability of the membrane of the corpuscle does not
increase steadily, but the injury ends with a kind of all-or none process,
during which the membrane passes from a state of high to one of
negligible resistance

Since it is assumed that the resistivity of the effective cells is high
as compared with that of the intercellular fluid, the values of C_m
and $1/R_m$ can be calculated by the method used for normal corpuscles
For this purpose we assume the corpuscles to be spherical, giving α
$= 1\,50$, and we assume furthermore that their volume is the same as it
was originally   By these assumptions, the diameter $2a$ is determined
The graphical representations of C_m and $1/R_m$, given in Fig 2, are for
the suspensions, for which data are given in Fig 6   The curves con-
firm the statement made as to the general similarity of the injury pro-
duced by saponin and by water   The fact that the constant value of
C_m, at high frequencies, is the same as that for the normal corpuscle,
lends justification to the theoretical treatment used

It may be assumed that the disintegration of the membranes of the
corpuscles is related to the gradual disappearance of ghosts **noted**

when increasing concentrations of saponin are added to a corpuscle suspension, and usually referred to as stromatolysis Although the two phenomena may not be identical, we may for the present designate them by the same term

The volume of the stromatolyzed corpuscles, as a percentage of the original volume of the corpuscles, is given by $100 \ (1 - \ \rho_o/\rho_i)$ We refer to this term as the "percentage stromatolysis " Fig 7 shows
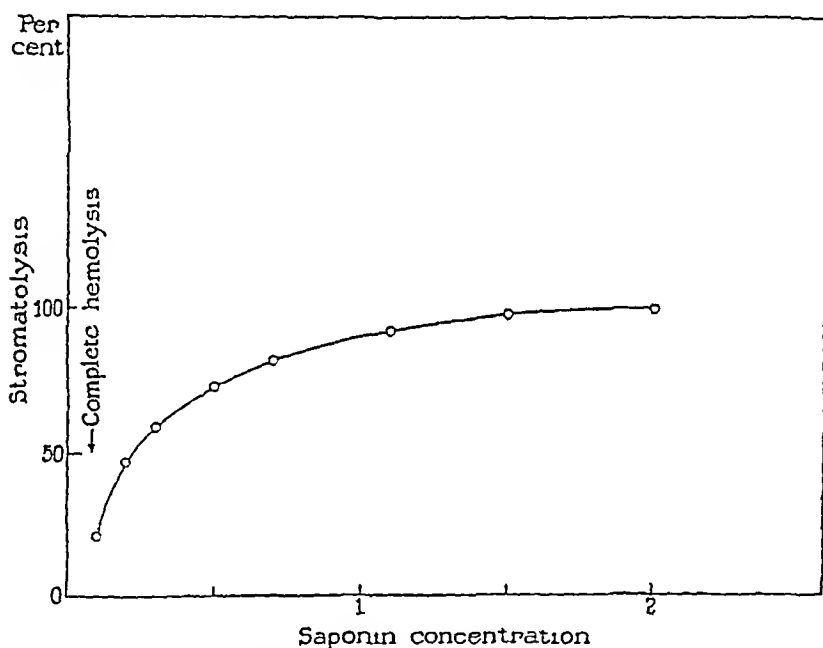
Fig 7 Percentage stromatolysis as a function of saponin concentration for a 40 per cent suspension of rabbit corpuscles in saline

the percentage stromatolysis as a function of the concentration of saponin, for a 40 per cent suspension of rabbit corpuscles in saline

Complement and Amboceptor, Sodium Taurocholate, Digitonin — The results obtained are in general the same as those for saponin Expressing the quantity of a lysin in terms of that required to produce 50 per cent lysis, it is found that the stromatolysis produced with complement and amboceptor is *considerably less than that* produced with the corresponding quantity of saponin   Stewart (8) noted that the electric resistance of a suspension of red corpuscles

bemolyzed with saponin was less than when lysis was produced with complement and amboceptor, and drew from this observation the conclusion that the pigment and the electrolytes were liberated each independently of the other   This conclusion is not justified since it does not consider the difference in stromatolysis, and we have found no indication of any such differential liberation of pigments and electrolytes
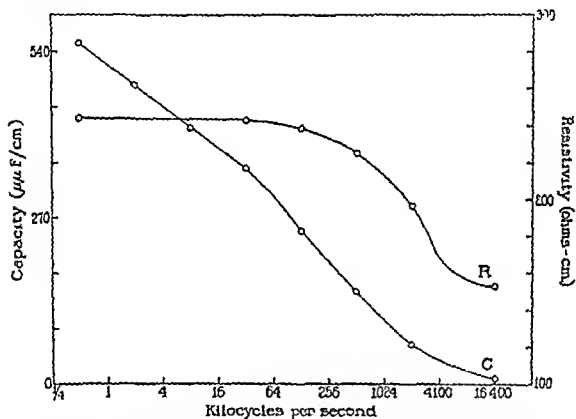
FIG 8 Resistivity (R) and capacity (C) for a suspension of rabbit corpuscles hemolyzed by freezing and thawing four times with carbon dioxide snow

Freezing and Thawing—In Fig 8 are given C and R, as functions of the frequency, for a 74 per cent suspension of rabbit corpuscles, frozen and thawed four times with carbon dioxide snow   The value of $\rho/\rho_1 = 0.43$ Repetition of the freezing leaves the general form of the curves for C and R unchanged, but the value of ρ_o/ρ_1 decreases We are doubtful about the interpretation of these curves, and have not attempted to derive the values of C_m and $1/R_m$ The form of the curve for C appears to indicate a distinct difference between lysis produced by freezing and thawing and by the other lysins used

SUMMARY

This paper is concerned with the changes in the electric surface capacity and surface resistivity of the membrane surrounding the mammalian red corpuscle, as a result of various types of hemolysis In the case of hemolysis with water, the cells swell with no apparent change in the electric properties of the membrane   They then hemolyze, but the membrane persists, although showing evidence of injury, as indicated by a change in the frequency dependence of its capacity and resistivity at low frequencies   The fact that a change of the frequency dependence takes place shows that the injury cannot be due merely to a rupture in the membrane, but must be due to changes in the properties (increased permeability) of the membrane as a whole

With chemical lysins (saponin, complement and amboceptor, digitonin, sodium taurocholate) a similar type of injury to the membranes of a certain number of the corpuscles takes place, to an increasing extent as the concentration of lysin is increased   The rest of the corpuscles become completely permeable to the electric current, and as the amount of lysin is increased, this number of completely permeable corpuscles increases until all are affected   This change, presumably associated with a disintegration of the corpuscle membrane, is referred to as stromatolysis, and the method gives a quantitative means of determining percentage stromatolysis

For lysis by freezing and thawing, the results obtained indicate this type of lysis to be different from that of the others studied

In its earlier stages, this work was carried out in collaboration with Dr H Goldblatt of Western Reserve University   For the greater part, we have had the benefit of the active interest of Dr E Ponder of this laboratory

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THE CHROMATOPHORAL NEUROHUMORS OF THE DOGFISH

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I

INTRODUCTION

The common smooth dogfish, *Mustelus canis*, has two extreme tints of skin, a light one due to the concentration of its melanophore pigment and a dark one resulting from a dispersion of this coloring matter The dispersion of the pigment is excited by a neurohumor produced in the pituitary gland and carried from that gland to the melanophores by the blood (Lundstrom and Bard, 1932) The concentration of this pigment is the result of specific, local, nerve action by nerve fibers in all probability from the autonomic system and sympathetic in origin (Parker and Porter, 1934) It is well known that the defibrinated blood of a dark dogfish when injected into a light one will call forth a temporary dark spot in the skin of the recipient (Table I, 16), but it will have no effect upon the skin of another dark fish Defibrinated blood from a light dogfish, however, is without effect upon the skin of either light or dark individuals (Table I, 15) If a neurohumor is produced by the concentrating nervous mechanism of the dogfish, it is not water-soluble, for, unlike the dispersing humor, it does not occur in the blood From work done on *Fundulus* and on *Ameiurus* (Parker, 1934 a, 1934 b) such a concentrating neurohumor has been demonstrated to exist and since this neurohumor is known to spread from place to place and yet is not carried in the blood (Matthews, 1933), it has been suggested that it is oil soluble and that its means of transfer is through the lipoid constituents of the integumentary cells (Parker, 1934 a)

Is there any evidence that such an oil soluble neurohumor exists in the dogfish or is the action of the concentrating nerve fibers in this animal of an entirely different nature? This question will be discussed in the following pages

II

Methods

In the smooth dogfish the parts of the integument that show the most pronounced changes in tint are the dorsal skin and especially the fins   It was soon found that of these two parts the fins were the more effective (Table I, 2, 4), hence in the majority of cases the fins only were used   They were removed from a dogfish that had been blanched to an extreme degree by having been kept for some 4 days or so in a white-walled tank illuminated from above by a strong electric light   They were then reduced to a pulp by being put through a kitchen pulverizer after which they were ground in a rough porcelain mortar for an hour or more with about 1 cc of pure Italian olive oil   The pasty mixture that resulted was then allowed to stand some 12 or more hours in a refrigerator for extraction and after agitation with a small amount of sea water, it was set aside to separate The oil rose to the surface, was decanted or skimmed off, and shaken thoroughly with the small amount of watery fluid that accompanied it   The emulsion thus produced was injected by means of a fine hypodermic syringe in known volume into the subcutaneous spaces of an appropriately tinted dogfish   The fish was then returned to a sea water tank and kept under observation

This general procedure was followed throughout most of these investigations In the early part of the work a number of the injections showed after some days clear signs of infection   Considering the way in which the emulsions were made and used, this was not surprising   In the latter part of the work sterilization was employed and under these circumstances little or no infection occurred   The procedure being such as it was (hypodermic injection through the wet skin of a dogfish with subsequent return of the fish to sea water) there was no possibility of excluding infection completely, but such disturbances were so greatly reduced in the later part of the work that they ceased to have any possible significance in the results   Extracted juices, olive oil, Ringer's solution, sea water and the like were sterilized by subjecting them to the temperature of boiling water for at least a quarter of an hour   Instruments were sterilized either by boiling them or by steeping them in alcohol

As a rule, four injections were made on each dogfish, two on either side of the anterior and of the posterior dorsal fins, each about midway on the flank between the dorsal line and the lateral line   The volume of fluid injected in each test was very regularly 0 5 cc   In this way a technique was developed that was believed to meet the requirements of the research

III

Observations

In all, twenty-five injections of non-sterilized oil extracts from light dogfish fins were made into over a dozen dark dogfishes (Table I, 1)

Many of these injections were followed almost immediately by one or more light spots in the region concerned   These spots were always temporary   Thus in one dogfish two non sterilized injections were made, one to the right and the other to the left of the posterior dorsal fin   5 minutes after this had been done a large, faint light spot over a centimeter in diameter began to appear in the region of the right hand injection   5 minutes after this three light spots, each a few millimeters in diameter, had made their appearance over the left hand injection 10 minutes later the large faint spot had mostly disappeared and 25 minutes after that or three quarters of an hour after the injections had been made, all three smaller spots had faded out   These initial temporary light spots, which may be called primary spots, were to be noticed in the great majority of injections   They never lasted over an hour or so and their disappearance was invariable   In my opinion these spots are purely operative in their origin for they may occur with injections of sterilized oil and sea water containing no extractives as well as with other fluids   They are due, I believe, to the mechanical action of the injected fluid itself on the invaded tissues   The injected fluid when it first enters the tissue is in such concentrated volume as to strain and thereby stimulate small nerves or possibly to press upon blood vessels to such an extent that small areas in the skin are temporarily cut off from the necessary blood supply   Both these means are known to produce concentration of melanophore pigment and consequently to blanch the skin   As the injected fluid slowly seeps out from the region of entrance into the adjacent tissues, the pressure on the containing tissues must be relieved with the result that nerves and blood vessels return more nearly to their normal states and the spots thus tend to disappear

If fishes of the kind that have been described are kept till after such primary spots have vanished, new, larger, and relatively permanent light spots may arise in them (Fig 1A)   These spots which may be called the secondary spots, are first visible in from 1 to 2 days after the injection has been made and remain commonly for several days after which they may gradually fade out   Of the twenty five injections of non sterilized oil extracts fourteen yielded secondary spots and eleven failed to show them (Table I,1)   The failures in these tests are attributed to unsuccessful injection technique, in inserting
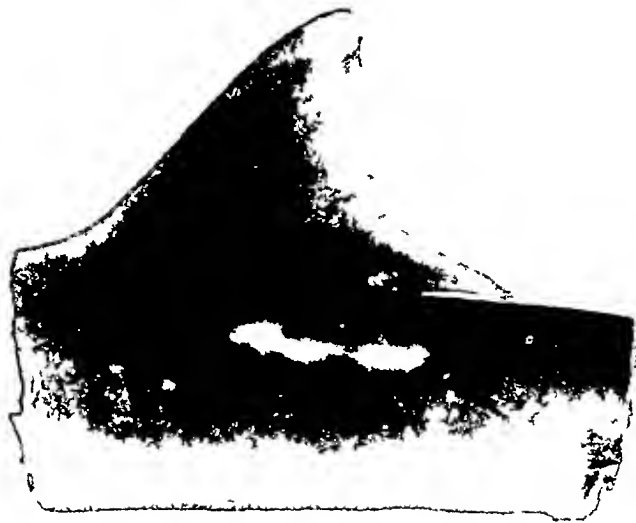
FIG 1 Part of the trunk of a smooth dogfish, *Mustelus canis,* in the region of the anterior dorsal fin

A Left side of the fish showing a secondary light spot due to an injection of 0 5 cc of an emulsion of olive oil extract of blanched fins and sea water made a little over a day previously   The injection was directed anteriorly from a point about 1 5 cm posterior to the posterior edge of the light spot   The preparation was made 26 hours after the first appearance of the light spot

B Right side of the same fish showing no change in color after the injection of 0 5 cc of an emulsion of olive oil and sea water made in the same way as the injection on the left side

the needle of the syringe the point of which was probably thrust, in the unsuccessful cases, too deeply into the muscle and was not slipped closely enough under the skin   In consequence the emulsion was discharged into deep seated spaces rather than directly next the inner surface of the integument and hence it failed to act effectively on the color cells   It is also possible that some of the earlier extractions were made with too much oil in relation to the amount of fin substance and

TABLE I

A summary of the collective reactions of the integumentary melanophores in the smooth dogfish *Mustelus canis*, to a variety of liquids introduced subcutaneously   The table includes records of the occurrence of dark spots, D S (dispersed melanophore pigment), of secondary light spots L S (concentrated melanophore pigment), of failure to react, N R., and of infection, Inf   The volume of liquid injected in each instance was 0 5 cc.

	D S.	L S	N R.	Inf
1 Light fins, oil extraction not sterilized	0	14	11	6
2 Light fins oil extraction sterilized	0	8	6	0
3 Light fins sea water not sterilized	0	0	4	0
4 Light skin oil extraction, not sterilized	0	0	2	0
5 Light skin, sea water not sterilized	0	0	1	1
6 Dark fins oil extraction not sterilized	0	0	2	0
7 Muscle oil extraction not sterilized	0	0	2	0
8 Oil and sea water,	0	0	6	1
9 Oil and sea water sterilized	0	0	2	0
10 Mercuric chloride 0 5 per cent	0	3	0	0
11 Formaldehyde 5 per cent	0	3	0	0
12 Light fins ether extraction oil	0	1	3	0
13 Light skin, ether extraction oil	0	0	3	0
14 Light fins Soxhlet extract, oil sterilized	0	4	0	0
15 Light blood defibrinated	0	0	3	0
16 Dark blood defibrinated	3	0	0	0

were in consequence too weak   Six of the twenty five injections showed obvious infections in that they developed large, swollen, red centers which sooner or later sloughed away

The injection of sterilized oil extract of light fins into dark dogfishes was made in fourteen cases of which eight showed secondary light spots and six no reactions (Table I, 2)   As might have been expected none of these fourteen cases showed infection

The question now to be considered is the nature of the secondary light spots It is well known that in death the skin of a dogfish finally blanches Is it possible that the methods of injection used lightened the skin of the experimental dogfish by producing local death? In other words, are not the secondary light spots dead areas in the integument of the fish? Such dead areas can readily be produced in dogfish skin by injecting solutions of mercuric chloride, 0 5 per cent, or formaldehyde, 5 per cent, immediately under the skin of the living fish Such a solution of mercuric chloride produced a large white spot in a dogfish in an hour and a half after injection and this spot remained permanently on the fish for 18 hours when death intervened (Table I, 10) Formaldehyde, 5 per cent, began whitening the skin of a fish some 2 hours after its injection and produced a pronounced light spot 3 hours later This also was a permanent spot (Table I, 11) When 1 cc of obstetrical pituitrin (Parke, Davis and Co) is injected into a light dogfish the fish turns quickly but temporarily dark When such an injection was made into a dogfish with a light spot from either mercuric chloride or formaldehyde, the whole fish turned dark except the light spot which remained unaltered Spots induced by these two reagents were not changeable in any way and gave every evidence of being composed of dead tissue When obstetrical pituitrin was injected into a dogfish with a light spot resulting from the injection of an oil extract of light dogfish fins, the spot became fully dark (like the rest of the integument of the fish) in three-quarters of an hour to return to a light tint $2\frac{1}{2}$ hours later I concluded from these tests that the oil extract of the fins does not kill the integumentary melanophores of the dogfish, but induces them to concentrate their pigment and leaves them in a fully active and responsive condition Hence the blanching of the skin in the dogfish by oil extract of light fins is not the result of the death of the melanophores

This blanching is also not due to the injected oil and sea water either unsterilized (Table I, 8, Fig 1 B) or sterilized (Table I, 9) nor is it produced by a non-sterilized oil extract of muscle (Table I, 7) It is to be regretted that there was no opportunity to extract other tissues, but certain aspects of the work took so much time that when this topic was reached the dogfish season was so far advanced that sufficient material was no longer available

Other methods of extraction than by olive oil were likewise used Plain sea water extract of light fins (Table I, 3) and of light skin (Table I, 5) failed to produce light spots as might have been expected from the fact that the blood of a light dogfish when injected into a dark one was without effect on the melanophores Ether was also used as a means of extraction To avoid the complicating factor of heat, fresh, wet fin paste from a light dogfish was mixed directly with about 100 cc of pure ether and was agitated in a closed bottle from time to time over a period of some 15 days The ether was then decanted and filtered and the light straw colored liquid was allowed to evaporate On full evaporation there was left in the small beaker that contained the fluid a translucent, thick deposit In amount this was roughly 1 cc It was mixed with an equal volume of sterilized olive oil and this mixture was emulsified with 1 cc of sterile sea water Four injections were made with this emulsion One of them was followed by a secondary light spot and three showed no reaction (Table I, 12) A similar injection of ether extract of light skin excited no response at all (Table I, 13) Apparently ether is a means of extracting what blanching substance there may be in the fins As a source of this material the body skin appeared to be entirely unfavorable

As a result of these tests ether extraction was attempted with a Soxhlet apparatus I am indebted to Professor R Hober for many suggestions and much kindly help at this stage of the work The fins of two light dogfishes were dried in an oven at 110°C for 12 hours They were then ground to a powder and this powder was extracted with ether in a Soxhlet apparatus for 28 hours The apparatus ran with a turnover of about once in every 10 minutes The final result was some 50 cc of a straw colored ether deeper in tint than that which had been obtained by direct ether extraction This was fully evaporated after which a thick transparent deposit of material was left in the beaker, in all about 1 cc in volume This was mixed with 1 cc of sterilized olive oil after which the mixture was emulsified with 1 cc of sterilized sea water Four injections of this emulsion in two places each in two dark dogfishes were then made These fishes had been tested previously by having had cuts made in their fins to show that they could lighten locally Primary light spots appeared in all four places in about 15 minutes In 3 hours these had completely disap

peared 21 hours after the injections each of the four areas showed a secondary light spot All four spots were faint but easily noticeable 5 hours after their first appearance all the spots were well marked 1 cc of obstetrical pituitrin was then injected into one fish and 12 minutes after the injection the light spot nearer the region of introduction had lost much of its conspicuousness, half an hour later it had entirely disappeared 6 hours after the injection it again regained visibility Therefore, the conclusion was drawn that the fin substance that induces a concentration of the melanophore pigment is open to extraction by ether with a Soxhlet apparatus and that it is a material that is stable even after having been heated to 110°C The single Soxhlet extraction that was completed came unfortunately at the end of the dogfish season, and in consequence it was not possible to repeat it Such a test should be gone over many times and the extracts obtained should be subjected to fractionation with a view of isolating the substance or substances concerned in melanophore activation Because of the lack of dogfishes these steps in the work must be deferred till some time in the future

As the summary in Table I shows, true secondary light spots in the skin of dark dogfishes have been induced only by oil extracts (1, 2) or by ether extracts (12, 14) of light fins They have never been excited by oil extracts of dark fins (6) nor by water extracts of light fins (3) Extracts of light skin with oil (4), with ether (13), or with sea water (5) have never yielded secondary light spots Light spots are not produced by emulsions of oil and sea water (8, 9), by oil extract of muscle (7), nor by defibrinated blood from light or dark fishes (15, 16) Apparently some substance in the fins of the light dogfish, soluble in olive oil or in ether, but not in water, is capable of exciting the concentration of melanophore pigment This substance is believed to be a neurohumor produced by the concentrating nerve fibers of the melanophores, but it must be admitted that its origin is certain only in that it is from some part of the fin So far as my observations go, it may come from any part of that structure Nevertheless it is strongly suspected that it is of nervous origin Quantitative studies may eventually help in deciding the exact source Till some such step is taken this source must remain unknown It is, however, of no small interest from the standpoint of the neurohumoral hypothesis

(Parker, 1932) that in the dogfish the concentrating response which has all the appearances of a purely nervous one, as contrasted with the hormonal dispersing reaction, should be open to excitation by extractives of the kind described The whole situation seems to point to a system of neurohumors in which two well defined categories can be distinguished, one composed of substances like the pituitary extract which is soluble in water and hence open to quick carriage by the blood, and the other made up of materials that are soluble in oil and that pass only slowly from place to place probably through the lipoid components of the cells Such materials might be called liponeurohumors as contrasted with those soluble in water, hydroneurohumors In *Mustelus*, if this analysis is correct, darkening is due to a pituitary hydroneurohumor and blanching to a liponeurohumor from integumentary nerves In *Fundulus*, whose blood carries no neurohumor, both concentration and dispersion of melanophore pigment must be due to liponeurohumors whereas in the frog, whose blood is generally conceded to be the all important element, only hydroneurohumors appear to be present These results give some insight into the complexity of the neurohumoral system, a complexity which, however, is not beyond analysis

IV

SUMMARY

1 The common dogfish, *Mustelus canis*, as is well known, exhibits two temporary extremes of tint, one dark, the other light The dark phase is induced by a secretion from the pituitary gland which is carried in the blood, hence a substance soluble in water (a hydroneurohumor) The light phase is under the control of nerves and cannot be excited by blood from a light fish

2 When an olive oil or ether extract is made from the fins of a light dogfish and this extract is injected into a dark fish, large light spots may appear in from 1 to 2 days and persist for several days These light spots, which may be called secondary spots, are not to be confused with certain small and very temporary light spots, the primary spots, which occur soon after the injection and which are believed to be purely operative in origin

3 The secondary light spots are not due to the death of the integu

mentary tissue, for, after their formation, they can be made to disappear by the action of obstetrical pituitrin and will subsequently reappear

4 They are produced by some substance extracted from the light fins by ether or by olive oil   They are not produced by sea water, ether, or olive oil alone

5 The extracted substance, which can resist dry heat up to at least 110°C , owes its limited range of action in the dogfish to its inability to dissolve in water   It is soluble in oil (a liponeurohumor)

6 This liponeurohumor is believed to emanate from the nerve terminals concerned with the concentration of melanophore pigment and to spread through the fatty components of the integumentary cells

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CORRELATION OF THE VISCOSITIES OF PROTEIN SOLUTIONS WITH THEIR ABILITY TO CRYSTALLIZE

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Cohn (1925) was the first to suggest a relationship between viscosity of protein solutions and their ability to crystallize "Thus the proteins that are small and of high valence seem to be readily crystallizable, soluble and of low viscosity, whereas those that are large are very viscous, are only slightly soluble when uncombined with acids and bases, and have not yet been crystallized" Since 1925 more data have become available and it now appears that no simple relationship between viscosity and molecular weight or solubility exists but that the part of Cohn's generalization applying to viscosity and ability to crystallize is still valid

All those proteins, whose solutions at a given concentration yield viscosities equal to or less than that of serum albumin, can be readily crystallized provided they can be obtained in isolated condition   All those proteins with viscosities equal to or greater than denatured ovalbumin have been obtained only in the amorphous state [1] This divides all the proteins, whose viscosities have been measured, into two groups the crystalline—hemoglobin, pepsin, urease, trypsin, insulin, ovalbumin, and serum albumin, and the amorphous—denatured hemoglobin, denatured ovalbumin, pseudoglobulin, euglobulin, casein, and gelatin   Several other proteins, notably plant globulins, have been crystallized but their viscosities have been determined

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[1] By a crystalline protein is meant here one which separates from a super saturated solution in the form of microscopically visible crystals By amorphous state is meant merely a subdivision so fine that crystals cannot be seen in the microscope. All the crystallizable proteins can also be obtained in the amorphous state if precipitation is carried out rapidly The non-crystallizable proteins never yield crystals under any conditions

In order to compare the viscosities of the proteins, for which measurements are not available over the same range of concentrations, an empirical equation suggested by Kunitz (1925-26), has been used

$$\frac{n_s}{n_w} = \frac{1 + 0\ 5\ \phi}{(1 - \phi)^4}$$

where n_s is the viscosity of the solution, n_w is the viscosity of the solvent, and ϕ is an arbitrary term interpreted by Kunitz to be the fraction of the volume of solution occupied by the protein   This equation applies to the high viscosities of gelatin solutions and to the low viscosities of albumin solutions   It covers the whole range of concentrations and has the advantage of containing only one arbitrary constant   The fraction of volume, ϕ, occupied by the protein is approximately proportional to the concentration, C Thus ϕ/C is the implied arbitrary constant in the Kunitz equation   Values of ϕ/C are characteristic of the proteins and are reported in Tables I and II to indicate the magnitude of the viscosities of protein solutions

Fig 1 shows the effect of concentration on viscosity for the various proteins and their sharp division into two groups   The curves are the smooth curves of the Kunitz equation   From the experimental data for relative viscosity of each protein at a certain concentration, ϕ was obtained by reference to a graph of the Kunitz equation   It was assumed that for each protein ϕ/C was constant   Then from ϕ/C the relative viscosity at any concentration can be calculated   The following is a sample calculation   Serum albumin has a relative viscosity of 1 95 when the solution contains 10 79 gm protein per 100 cc solution   Reference to a graph of the Kunitz equation shows that the volume occupied by the protein equals 14 0 cc   Then $\frac{100\phi}{C}$ = $\frac{14\ 0}{10\ 79}$ = 1 30   Now for another concentration such as 6 0 per cent the apparent volume occupied by the protein would be 6 0 $\times$ 1 30 or 7 8 cc   A value of 100ϕ of 7 8 corresponds to a relative viscosity of 1 440   In this way values of relative viscosity for concentrations below 10 per cent have been found for all the proteins

It can be seen from the graph that for a concentration of 1 per cent the crystallizable proteins have a relative viscosity less than 1 060

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TABLE I
Relative Viscosity of the Non Crystallable Proteins

Protein	Concentration solution C	Relative viscosity $\frac{\eta}{\eta_w}$	$\frac{100\phi}{c}$	References
	gm. per 100 cc			
Gelatin 35 C	4 0	4 24	7 0	Kunitz (1926–27)
Casein 25 C	4 35	3 37	5 5	Chick and Martin (1912)
Euglobulin 25 C	6 88	3 32	3 5	Chick (1914)
	3 0	1 572	3 2	Loughlin and Lewis (1932)
Denatured hemoglobin 25 C	0 2	1 026	3 0	Loughlin and Lewis (1932)
30 C	2 67	1 6	3 75	Anson and Mirsky (1931–32)
	3 64	1 40	2 0	Chick (1914)
Pseudoglobulin 25 C	6 03	1 85	2 14	Chick (1914)
Denatured ovalbumin 25 C	3 0	1 303	1 91	Loughlin and Lewis (1932)

TABLE II
Relative Viscosity of the Crystallable Proteins

Protein	Concentration solution C	Relative viscosity $\frac{\eta}{\eta_w}$	$\frac{100\phi}{c}$	Reference
	gm. per 100 cc			
	10 79	1 95	1 3	Chick and Lubrzynska (1914)
Serum albumin 25 C	4 26	1 29	1 3	Unpublished results in this laboratory
Urease 0 C	4 0	1 263	1 27	Kunitz Anson and Northrop (1933–34)
Trypsin 5 C	9 08	1 57	1 06	Chick and Lubrzynska (1914)
	15 1	2 21	1 08	Chick and Lubrzynska (1914)
Ovalbumin 25 C	3 0	1 168	1 13	Loughlin and Lewis (1932)
	1 0	1 048	1 0	Gerlough and Bates (1932)
	10 0	1 596	1 0	Gerlough and Bates (1932)
Insulin	4 2	1 175	0 84	Kunitz Anson and Northrop (1933–34)
Hemoglobin CO 5 C	5 4	1 129	0 8	Unpublished results in this laboratory
Pepsin 0 C	1 7	1 067	0 8	
	0 85	1 036	0 9	

while the amorphous proteins have a relative viscosity greater than 1 085 Or, stated in another way, when a protein occupies an apparent volume in solution less than 1 3 times its concentration it belongs to the crystallizable group of proteins The amorphous proteins have an apparent volume in solution greater than 1 91 times their concentrations Unfortunately different workers often disagree as to the viscosity of a given protein The cause of this lack of reproducibility is still unknown It seems likely that proteins which have
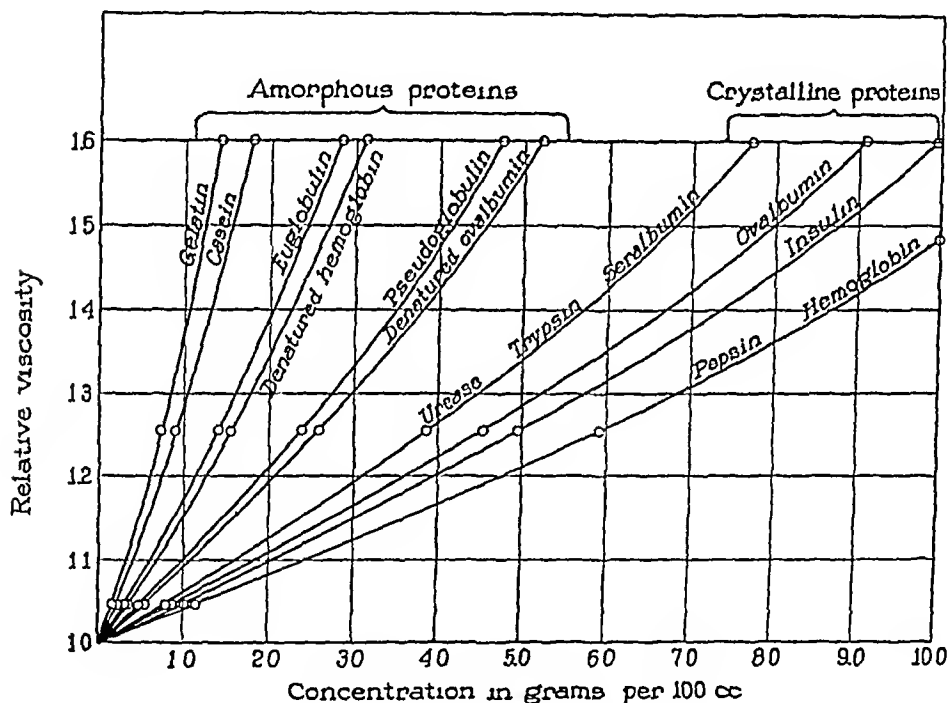
Fig 1 Viscosities of protein solutions

been subjected to prolonged dialysis have abnormally high viscosities The lowest reported figures for viscosities are likely to be correct Furthermore the viscosities of the more viscous proteins vary considerably with pH and salt concentrations However, these effects are not large enough to make any difference with respect to the classification of the proteins into two groups

Our knowledge of the factors influencing the viscosity of protein solutions is still far from complete Staudinger's (1932) work on

hemicolloids suggests that molecular weight must be taken into account Anson and Mirsky (1931–32) have shown that any factor that tends to aggregate the protein will increase viscosity Equations proposed by Einstein (1905, 1911), Hatschek, (1910, 1911, 1912) and Arrhenius (1917) disregard all other factors except the volume of the protein in solution which is presumably increased by solvation Loeb (1924) explained the viscosity of gelatin sols as due to minute particles of gel in suspension Northrop and Kunitz (1925–26) have postulated the existence in gelatin solutions of two forms of gelatin one form occurring as insoluble micellae, and the other a soluble form distributed between the micellae and the outer solution Kunitz (1926–27) has accounted for the high viscosity of gelatin and also its variation with pH on this assumption of heterogeneous solutions Eisenschitz (1931–32, 1932–33) has derived a theoretical equation which relates viscosity not only to the volume of the colloid in solution but also to the length and thickness of the molecules Using this equation Schulz (1932) has calculated the length and thickness of gelatin molecules

The correlation between viscosity and ability to crystallize is an empirical fact which should be of importance in the final development of a theory of protein solutions

I wish to express my obligation to Professor J B Sumner for the preparation of crystalline pepsin and for valuable advice and criticism

SUMMARY

All the proteins whose viscosities in solution have been measured fall distinctly into two classes Proteins of viscosity equal to and lower than serum albumin are readily crystallized Proteins with viscosity equal to or greater than denatured ovalbumin have not yet been crystallized

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THE EFFECT OF LIGHT INTENSITY, AREA, AND FLICKER FREQUENCY ON THE VISUAL REACTIONS OF THE HONEY BEE

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I

Experimental studies on the visual capacity of the eye of the honey bee have shown that the relative motion of the eye and the object seen plays an important rôle in vision Bees show an increased sensitivity toward moving objects and flickering visual stimuli based upon the frequency of intermittent stimulation of the elements of the ommatidial mosaic of the retina (Wolf, 1932–33 *a*, *b*, Wolf, 1933–34, Wolf and Crozier, 1932–33, Wolf, 1933, Zerrahn, 1933) It was found that the bee's reaction depends upon the light intensity, the velocity of motion of the test object or the flicker frequency, and the size of the object It seemed to be essential to obtain quantitative relationships between these factors For this investigation the phototropic response of the bee was used

II

The experimental set up can be understood with the help of Fig 1 In a vertical piece of ply wood 175 cm long and 50 cm high two openings 37 × 37 cm were cut The distance between the centers of the two holes is 64 cm Into each opening opal glass plates are fitted which are illuminated from behind The areas of the illuminated opal plates can be varied by means of cardboard frames, reducing the original area to ½, ¼, ⅛ and ⅟₁₆ The cardboard frames were constructed so that their lower edges coincided with the lower edge of the opening in the ply wood so that the illuminated fields were always at eye level ' of the bees

The vertical wooden wall serves as one side of a cage, triangular in shape in which the bees are allowed to migrate to the illuminated fields The cage is 170 cm in length the bottom consists of cardboard the sides and top of wire screens, through which the bees can be observed The bees are admitted through a door at the distal end of the cage they can after arriving at the illuminated fields be taken out through side doors in the cage

For illumination of the opal plates in the wall of the cage two 500 watt concentrated-filament lamps are used   To provide an even illumination of both areas the lamps are set back 150 cm   Directly in front of each source a small opal plate is placed which provides a larger and more easily controllable source of uniform illumination than the lamps   The amount of light emitted from these diffusing screens is controlled by diaphragms which permit a variation in brightness over a range of 200 units   The brightness of the large opal plates in the wall of the cage at different diaphragm openings is measured with a Macbeth illuminometer   From the photometric readings calibration curves are drawn from which any desired brightness can be read and the diaphragms adjusted accordingly
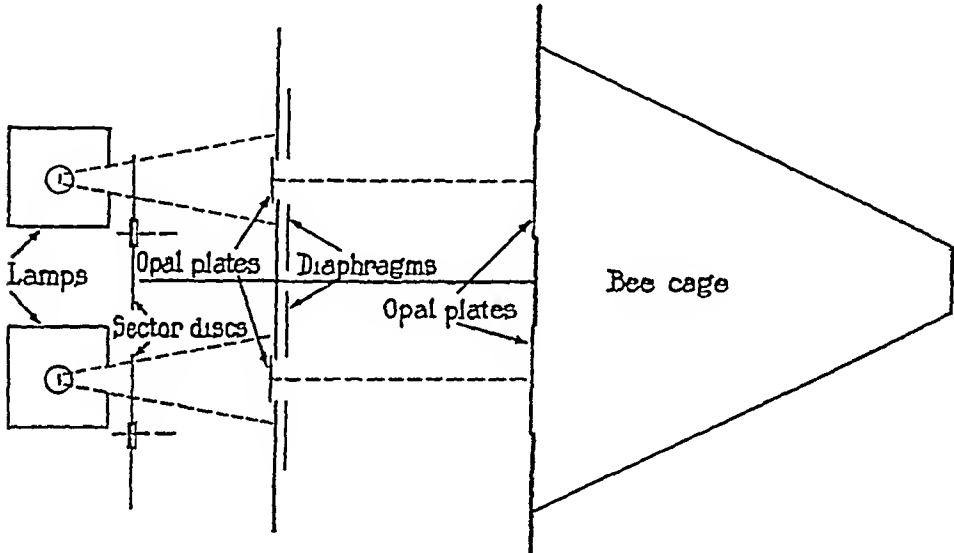
Fig 1 Apparatus for testing the bee's reaction to two illuminated fields differing in area, intensity, and flicker frequency

The experiments are carried on with single individuals   They are caught in front of one of our hives, left in the dark a short time for adaptation, and then set free at the narrow end of the cage   Being positively phototropic they move, in part crawling in part flying, toward the lights, and by changing the areas and their brightnesses one can easily recognize the stimulating effect of the two areas from the course of the bees' path   If the two areas are equal in brightness the course of the bees is generally the bisecting line between the two fields, and this is maintained until they almost reach the front wall, then swinging motions occur until the animals finally go to one of the two fields   If the two areas are not equal in their stimulating effect, the course of the bees is slightly turned to the brighter field   The angle of deflection from the vertical can serve as an indication of the difference between the stimulating values of the two fields

In the first series of experiments both fields were equal in intensity and size (37 × 37 cm) In a great number of tests equal numbers of bees travelled to both fields We now changed the relative areas of the two fields, keeping the intensities the same Testing in each case 50 bees and decreasing the size of one of the areas to 1/2, 1/4, 1/8, and 1/16 of the original, we obtained with increasing difference in area
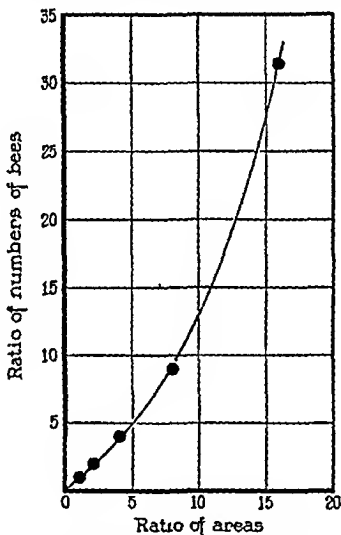
Fig 2 Relation between the relative numbers of bees seeking two illuminated fields and the relative sizes of the areas

an increasing proportion of animals going to the larger field The data are represented in Fig 2 where the relative numbers seeking the two fields are plotted against the relative areas

It may be assumed that the stronger response to the larger field at equal intensity is due to the greater number of elements stimulated, and hence the greater total frequency of retinal responses How

ever, Hartline and Graham (1932) have shown that the total frequency of optic nerve discharges in *Limulus* also increases with intensity   It therefore might be possible to equalize the stimulating effect of two different areas by decreasing the intensity of the larger area, until the total frequency of response from both areas is equal

To test this point we presented two different areas, then decreased the brightness of the larger field step by step until we obtained in a considerable number of trials equal numbers of bees going to both fields   At this point the product of area and intensity was found to be identical for both sides   The data are given in Table I

Since the larger area stimulates a greater number of retinal elements weakly, while the smaller area stimulates a smaller number more

TABLE I

Values for areas and intensities of illuminated fields which have the same stimulating effect upon the bee's eye   The products of area and intensity are approximately equal for two fields presented simultaneously

Area	Intensity, millilamberts	Area × intensity	Area	Intensity, millilamberts	Area × intensity
cm			*cm*		
1354 2	6 42	8696 9	1354 2	6 42	8696 9
681 2		4374 5		3 26	4304 8
338 6		2174 2		1 70	2302 2
169 0		1085 3		0 75	1019 7
86 5		555 4		0 54	578 9

strongly, due to the respective brightnesses, and the net effect is the same for the orientation of the bees, we must assume that it is irrelevant for the processes which control the coordination of motion whether weak impulses travel from a greater number of receptor elements over a greater number of nerve fibers, or whether a smaller number of stronger impulses pass over fewer pathways

III

In the experiments just mentioned continuous lights were used   The peculiar effect of intermittent light can be demonstrated by presenting to the bees simultaneously a stationary and a flickering field of the same objective intensity   If bees are exposed to two such

fields they practically always travel to the flickering field, provided that the flicker frequency is below fusion   Even if the light intensity or the frequency of the flickering field is decreased considerably it always shows a pronounced predominance (Wolf, 1933)

To obtain quantitative data upon the effect of flicker on the orienting reaction of bees with fields of different areas we placed in front of our light sources sector discs which were slowly rotated by a motor and gave flicker frequencies between 2 and 16 per second   One field was flickered at a constant frequency, the other at an integral multiple of this frequency   The question was whether such fields can be made equal in their stimulating effects by adjusting their areas accordingly

In the first experiment both fields were made equal in intensity and size (37 × 37 cm), the flicker frequency of one being twice that of the other   The bees now migrated to the field of higher frequency   We then decreased the size of this field and observed that as soon as its area was reduced to one half equal numbers of bees travelled to both fields   We then changed to different sizes of areas and flicker frequencies and found that under all circumstances a ratio of 1 2 in flicker frequency can be balanced by a reciprocal ratio of 2 1 in area   If flicker frequencies are produced in the ratio 1 4 balancing occurs when the relative areas are as 4 1 and so on   In all such tests it can be shown that to produce equal photic stimulation by flickering fields the flicker frequencies must be inversely proportional to the field areas   For phototropic orientation of bees we therefore have to assume that it is irrelevant whether a great number of photic receptors is stimulated at a low frequency or a smaller number more often per unit time

<div align="center">IV</div>

Since it was shown that the frequency of alternate stimulation of the elements of the ommatidial mosaic of the bee's eye is the basis for reaction to flickering fields, evidence has been brought forward that the same holds true for the bee's reaction toward patterns of different design in free choice experiments (Zerrahn, 1933, Wolf, 1933)   When conditioning bees for patterns we are not dealing with any visual impressions (learning) and recognition of known forms but with a forced reaction to the pattern   The reaction depends upon the

stimulation given by the contours of a pattern which cause transitions of the retinal elements from one state of excitation to another during the bee's flight over the pattern

From some unpublished experiments by Zerrahn we know that the choice is always in favor of a pattern greater in area, if two patterns of the same degree of coarseness are presented to the bees We can deduce from this that for the bee's reaction not a single retinal element but the total number which undergo changes in their state of excitation has to be taken into account Thus the question arises whether two patterns different in area can be made equal in their stimulating effects by adjusting the "grain" of the patterns, since by this means both might give the same number of alternating stimuli, causing the bees to go to one pattern just as often as to the other

To test this we used a method which was applied previously by Zerrahn Bees are conditioned to come to a table, covered with a glass plate on which they are fed with a sugar solution Underneath the glass plate is a white surface on which can be placed patterns for choice During feeding no patterns are shown because all choices are supposed to occur without previous conditioning but spontaneously After a large number of bees comes regularly to the table, the food is removed and two patterns presented The bees then settle down on one of the two patterns in a big bunch By means of a feather they are brushed off the glass plate The glass is cleaned quickly with a moist cloth to remove odors, then the figures are shifted in relative position to each other to avoid conditioning to any particular location on the table Then the bees are given a new chance to choose This can be repeated half a dozen times As soon as the number of searching bees decreases, sugar solution has to be given anew

For patterns, checkerboards and stripes were used Patterns of concentric design such as sectors, *etc* were not taken into consideration since they are complicated by the greater density of contour toward the center, which can hardly be treated analytically

A checkerboard pattern with checkers 1 cm square and a total area of 10 × 10 cm (A) is presented with other patterns of coarser grain First we present our pattern (A) together with a checkerboard with 2 cm squares and 10 × 10 cm area (B) In 51 tests the finer pattern is chosen 31 times, the rough pattern 11 times, and in 9 cases we obtained

collections of bees on both patterns The relation of choices of the finer to the coarser pattern is 2 8 1

The question now arises how much the size of the coarser pattern has to be increased until we obtain a 1 1 relationship of choices In analogy to our darkroom experiments we double the area and obtain a pattern (C) 14 × 14 cm in size and find that the relation in choices of (A) (C) becomes 1 1 In 54 trials pattern (A) is chosen 21 times, (B) 21 times, and in 12 cases we obtain choices of both patterns at the same time By increasing the size of the coarser pattern still further, using a pattern (D) 20 × 20 cm in size, the relation of choices is shifted in favor of the coarse pattern In 53 tests (A) is chosen 14 times, (D) 30 times, and in 9 cases bees collect above both patterns The relationship (A) (D) is now 1 2 1

In a second series of tests we combine pattern (A) with checkerboards with 1 5 cm squares First this is tried in an area of 10 × 10 cm (E) Since the areas of both patterns are the same we should expect from our previous results that the finer design takes predominance We obtain in fact in 54 tests 29 choices of pattern (A) and 18 of pattern (E) In 7 cases both are visited by bees In order to obtain a 1 1 relationship in choice with a pattern which has 1 5 cm squares we must use a pattern (F) which is 12 × 12 cm in size Then among 53 choices 26 are in favor of (A), 26 in favor of (F), and one remains undecided

Finally we present together with pattern (A) a still coarser design with 4 cm squares First we use a 10 × 10 cm field of this pattern (G) (A) now has a very pronounced overweight In 75 tests (A) is chosen 53 times, (G) 16 times, and in 6 cases (A) and (G) at the same time The relation (A) (G) now is 3 3 1 Increasing the size of (G) until we obtain a 1 1 relationship of choices we have to use a pattern 20 × 20 cm in size (H) In 60 tests we obtained 27 choices of (A), 27 choices of (H), and 6 choices of both together

The results of these experiments show that coarseness of design and area can be successfully combined to obtain equal stimulating values for different patterns The increase in area of the coarser pattern necessary for equal stimulating effect is directly proportional to the increase in size of the squares with 1 5 cm squares the area is 1 5 times that of pattern (A), with 2 cm squares twice that of (A), and

with 4 cm squares 4 times that of (A)   The necessitated increase in size can be explained with help of our statement made previously, namely, that the length of contours of the pattern is decisive for the bee's reaction, providing a measure of the number of elements undergoing changes in their state of excitation   We thus ought to expect that in all cases in which we obtain a 1 1 ratio in choice the lengths of contours of the patterns used are approximately identical (Table II)

Thus by two patterns presented simultaneously the same numbers of retinal elements must have experienced changes in their state of excitation   When the pattern is small and has a fine design, the elements stimulated within the eye during transition lie close together

TABLE II

Patterns which are different in area and coarseness of design but have the same stimulating effect upon the bee's eye   All patterns have about the same lengths of contours

Pattern	Area	Length of contours
	cm	*cm*
(A) 1 cm²	10 x 10	200
(B) 2 cm²	14 x 14	200
(Γ) 1 5 cm²	12 x 12	192
(H) 4 cm²	20 x 20	208

When the pattern is larger in area and coarser in design the elements stimulated lie further apart, covering a greater retinal area

Checkerboards must without any doubt give most easily the results theoretically asked for, since because of their structure they produce a very regular alternate stimulation of the retinal elements, regardless of the direction in which the patterns are crossed during flight   Conditions might be less favorable when using stripes, but in a great number of tests the chances must become even that the bees cross the patterns in the direction of the stripes or at 90° to them, as our experimental results show

For the test we combine a pattern (I), having stripes 1 cm in width, an area of 10 × 10 cm , and a length of contours of 110 cm with patterns having stripes 2 cm wide but different in area   First we combine (I) with a pattern (K, 2 cm stripes) which has an area of

10 × 10 cm In 53 tests (I) is chosen 31 times, (K) 17 times, and both patterns at the same time in 5 cases The relation of (I) (K) is 1 8 1, the relation of the lengths of contours is 110 72 cm We then increase the area of the coarser pattern until its length of contours corresponds to that of (I) The new pattern (L) has an area of 12 5 × 14 cm and a length of contours of 116 cm (I) and (L) are presented to the bees 50 times (I) is chosen 23 times, (L) 23 times, and in 4 cases bees collect above both patterns The ratio of choices is 1 1 which was

TABLE III

Summary of the Results of the Bees' Choices among Patterns Different in Area and Coarseness of Design

Exp No	No tests	Pattern				Pattern					Ratio fine coarse
		Checker boards	Area	Length of contours	No of choices	Checker boards	Area	Length of contours	No of cho ces	Choice of both patterns	
			cm	cm			cm	cm			
I	51	1 cm² (A)	10 × 10	200	31	2 cm² (B)	10 × 10	104	11	9	2 8 1
II	54	1 cm² (A)	10 × 10	200	21	2 cm² (C)	14 × 14	200	21	12	1 1
III	53	1 cm² (A)	10 × 10	200	14	2 cm² (D)	20 × 20	400	30	9	1 2 1
IV	54	1 cm² (A)	10 × 10	200	29	1 5 cm² (E)	10 × 10	142	18	7	1 6 1
V	53	1 cm³ (A)	10 × 10	200	26	1 5 cm² (F)	12 × 12	192	26	1	1 1
VI	75	1 cm² (A)	10 × 10	200	53	4 cm² (G)	10 × 10	64	16	6	3 3 1
VII	60	1 cm² (A)	10 × 10	200	27	4 cm² (H)	20 × 20	208	27	6	1 1
		Stripes				Stripes					
VIII	53	1 cm (F)	10 × 10	110	31	2 cm (K)	10 × 10	72	17	5	1 8 1
IX	50	1 cm (I)	10 × 10	110	23	2 cm (L)	12 5 × 14	116	23	4	1 1
X	52	1 cm (F)	10 × 10	110	15	2 cm (M)	20 × 20	220	32	5	1 2 1

to be expected from our considerations In one further experiment we combine (I) with a pattern (M) having an area of 20 × 20 cm and a length of contours of 220 cm Since for pattern (M) the length of contours has become the double of (I), its stimulating value should be increased Of the 52 tests made 15 are in favor of (I), 32 in favor of (M), and in 5 cases both patterns are chosen The ratio (I) (M) is 1 2 1

For comparison all the data for the experiments with patterns are brought together in Table III

V

A picture of the effect of the alternate stimulation upon the bee's eye during motion when looking at a pattern can be provided by mapping out on a sheet of translucent coordinate paper the points of intersection of the axes of the ommatidia with a plane whose distance from the eye corresponds to the distance of the pattern at the moment of choice   This distance is in our case approximately 15 cm   Such a system of coordinates can be obtained from a figure given by Baumgartner (1928) in his paper where he gives a schematic picture of the resolving power of the bee's eye   His coordinates only need to be replotted for our distance   By placing our patterns underneath the map of the eye one can count the number of elements which are covered by the white and by the black parts of the pattern   If now the pattern is moved over a unit length of distance in any direction the number of elements undergoing transition during the shift may be counted   For any coarser pattern which does not provide as many transitions as a fine one of the same area when moved over the same distance, the increase in size can be estimated which would give it a stimulating value corresponding to that of the fine pattern   Our investigations, in fact, began by studying the effect of patterns upon the elements of this model of the bee's eye and the calculations made were justified by the results of our experimental tests

SUMMARY

1   For the phototropic reaction of bees, the stimulating effects of two illuminated fields differing in intensity and area become equal when the product of area and intensity is the same for both fields

2   The effect of two areas differing in size and flicker frequency is the same for the bee, when the product of area and flicker frequency is equal for both fields

3   If two patterns of the same character but varying in size and coarseness are presented to bees for free choice, a 1 1 ratio of choices is obtained when both patterns stimulate equal numbers of retinal elements alternately by transition from one state of excitation to another

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THE VALIDITY OF TALBOT'S LAW FOR THE EYE OF THE HONEY BEE

By ERNST WOLF AND GERTRUD ZERRAHN WOLF

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I

For the human eye an illumination which is interrupted with sufficiently high frequency appears continuous The brightness of such a field corresponds to the objective intensity multiplied by the fraction of time during which the light reaches the observer Consequently a reduction in this fraction is equivalent visually to a corresponding re duction in light intensity (Talbot's law)

The validity of Talbot's law for the human eye has been proved and much discussed by a large number of investigators and theoretical explanations have been proposed for it based upon the photochemical processes underlying vision (for references see Hecht and Wolf, 1931–32) Besides those upon man, data are available for *Daphnia* (Ewald, 1913), the blow fly larva (Patten, 1914), for the larvae of barnacles and *Limulus* (Loeb and Northrop, 1917 and 1922–23), for *Mya arenaria* (Hecht and Wolf, 1931–32), and for several insects (Dolley, 1923, Mast and Dolley, 1924) The data are sufficient to assume the general validity of Talbot's law throughout the animal kingdom

The effect of intermittent photic stimulation of low frequency upon the eye of the honey bee has been pointed out in previous papers (Wolf, 1932–33a, b, Wolf, 1933–34, Wolf and Crozier, 1932–33, Zerrahn, 1933, Wolf, 1933, and Wolf and Zerrahn Wolf, 1934–35) The bee's reaction depends in all cases upon the frequency of alternate stimulation of the ommatidia We know that the photic response in creases with the number of changes in state of excitation of the retinal elements We found that the maximum frequency which can be per ceived by the bee is about 55 per second (Wolf, 1933–34) To test the critical flicker frequency a system of sectors was rotated underneath

the bee's creeping cage and its deviation from a straight course taken as an indication of the reaction

If two fields equal in brightness, one illuminated constantly and the other by intermittent light, are presented to the bees and they are allowed to move freely over a longer distance toward the two fields, we can test Talbot's law by means of the bee's positive phototropic response   At low flicker frequencies the flickering field has a greater effect   If Talbot's law should hold for the bee, we ought to expect that above the critical frequency both fields have the same stimulating value, when equal in brightness
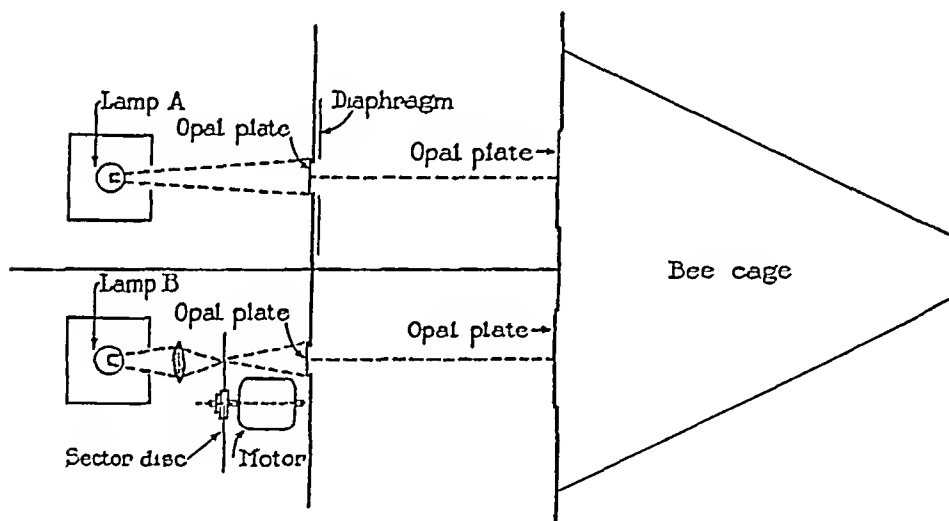
FIG 1 Apparatus for the test of the validity of Talbot's law for the eye of the honey bee

II

To test the validity of Talbot's law for the eye of the bee two square openings 25 × 25 cm are cut into a wall of ply-wood (Fig 1)   The distance between the centers of the two openings is 50 cm   Into each one an opal glass plate is fitted to which the bees react phototropically, when illuminated from behind   To illuminate each field a separate light source is used, consisting of a 500 watt concentrated filament lamp   The distance of the lamps from the screens is 150 cm   To provide a uniform illumination of each field a second set of small opal plates is placed in front of the lamps, providing a larger and more easily controllable source of uniform illumination than the lamps   In front of light source *A* is a diaphragm which permits a wide range of variation in brightness of the illuminated,

large opal plate In front of source B sector discs are rotated by a motor at a speed of 3600 R.P M The sector discs used were made of sheet aluminum. Each one had two sectors cut out on diametrically opposite sides, varying in angle between 5 75 and 160° With a speed of 3600 R P M the flickering field looks to be uniformly illuminated and its brightness can easily be measured for each size of sector For source A the diaphragm was calibrated and a calibration curve plotted From this curve any diaphragm setting could be read with sufficient accuracy so that both fields could be made photometrically equal

To the vertical wall with the two illuminated squares a cage is attached which is triangular in shape and whose apex is 170 cm from the wall For the test both

TABLE 1

Numbers of bees going to a flickering and a stationary field which are photometrically equal.

	Flickering field			Stationary field	
Sector	I millilamberts	Transmission	No of bees	I millilamberts	No of bees
degrees		per cent			
5 75	0 113	2 75	28	0 118	30
11 25	0 275	6 36	24	0 275	26
15	0 352	8 14	23	0 351	27
22 5	0 519	12 01	24	0 516	26
30	0 70S	16 40	26	0 70S	24
45	1 14	26 33	25	1 14	25
90	2 16	49 92	25	2 16	25
120	2 95	68 25	26	2 95	24
135	3 35	77 44	23	3 33	27
150	3 77	87 29	24	3 78	26
160	3 97	91 55	27	3 98	23
180	4 32	100 00	25	4 32	25

fields are made equal in brightness A bee is placed in the cage at its narrow end and its course observed In each trial only a single bee is set free to avoid any mutual disturbance The bee now moves partly crawling partly flying toward the illuminated fields Its course is the bisecting line between the two fields This course is kept until it almost reaches the front wall then zig zag movements occur and the chances of going to the field on the right or on the left are equally high

For each of eleven sectors we took records of the path of about 50 bees If Talbot s law holds for the bee, we would expect that equal numbers of bees go to the flickering and to the stationary fields This was found to be true within the limits of error for all sectors and intensities used

The results of the tests are best presented in Table I

SUMMARY

By presenting to bees two illuminated fields, equal in brightness, of which one is flickering and the other stationary we find that on account of the bee's positive phototropic response equal numbers of bees travel to both fields   We thus can assume that Talbot's law is valid for the eye of the bee

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THE EFFECTS OF RADIATIONS ON BIOLOGICAL SYSTEMS

III The Effect of Ultraviolet Light on the Respiration of Drosophila Larvae and the Duration of Their Prepupal Period

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In previous communications[1-3] a respirometer has been described which depends upon measurement (with adjustment for pressure and temperature) of change in refractivity and volume of the gas in a closed system in which the oxygen and carbon dioxide might vary independently but all other gases were present in either fixed amount or concentration, and the initial composition of the system known approximately. The system was provided with a means of circulation at a rate variable at will, and of regulation of temperature and humidity. The gaseous system was unaffected by the analysis except that circulation was temporarily stopped and a faint beam of light passed through a small portion in an attached interferometer while the bands were adjusted for readings—about 1 1/4 minutes were required for all adjustments and observations, five independent interferometer readings to the *nearest tenth* of a small scale division being made in quick succession. Chambers containing a respiring system could be attached and mercury sealed to the apparatus or detached readily. The object was to investigate variations in CO_2 respiratory rate of *Drosophila* larvae following x ray irradiation[4] or similar phenomena where the mean effect on large numbers of individuals could be observed directly. By means of a careful preparation of a large *batch* of individuals of which random samples could be taken for various treatment, it

[1] Thompson W R, *J Gen Physiol* 1932-33 16, 5
[2] Thompson W R, and Tennant R, *J Gen Physiol*, 1932-33 16, 23
[3] Thompson W R, and Tennant R *J Gen Physiol*, 1932-33 16, 27
[4] Hussey, R and Thompson, W R *J Gen Physiol*, 1934-35, 18, 669

was hoped that precise *mass* phenomena might be observed   Thus a lower CO_2 respiratory rate, followed by a rise (but not enough to again approximate the rate of controls) was found immediately after x-ray irradiation of larvae [4]

In earlier work[5-7] the increase in duration of the larval stage after x-ray irradiation had been studied, but we were unable to produce similar effects with ultraviolet light   Striking resemblance has been found, however, in effects of these agents upon respiration   The purpose of the present communication is to report some of the results obtained with ultraviolet irradiation under various conditions

Technique

The source of radiations for the present experiments was a mercury arc in quartz used in former work,[8] operated at an angle of 30° from horizontal   Larvae were irradiated in tumblers (as in the x-ray experiments[4]) about 61 cm below the lamp, and in a stream of air at approximately 22°C   They were prepared and maintained for respiratory studies on food of the same formula in the same manner in tapering glass tumblers as has been described[4] previously, except in certain experiments already reported[9] where variation of the sensitivity of the system to ultraviolet light was produced by increase in the acetic acid concentration in the food   About 200 to 500 larvae were used in each experimental lot, the lots within any given experiment being about equal in number

As in previous work,[4] the main interest was in estimation of relative respiratory rates of variously treated lots of larvae without unnecessary delay, and the same technique was employed with 20 minute observational intervals   Under the conditions of observation, the change in refractivity (Δr) of the inclosed gas was $2\,00(10)^{-8}\,\Delta y$ by calibration,[1] where Δy was the difference in interferometer readings   The increment in CO_2, previously[1] denoted by $'\Delta''Q_3$ in a general formulation, may be estimated from a simple formula obtained from relation (16) of the first mentioned paper[1] by substitution of $'\Delta''Q_3(1 - \frac{1}{F})$ for its equal, $C\,'\Delta''V$, where F is the respiratory quotient   Thus, dropping the primes, and introducing the values given by Edwards[10] for the various constants involved,

[5] Hussey, R , Thompson, W R , and Calhoun, E T , *Science*, 1927, 66, 65

[6] Tennant, R , *Science*, 1931, 73, 567

[7] Hussey, R , Thompson, W R , Tennant, R , and Campbell, N D , *J Gen Physiol* , 1932–33, 16, 207

[8] Thompson, W R , and Hussey, R , *J Gen Physiol* , 1931, 15, 9

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$$(1) \qquad \Delta Q_2 \cong \frac{V \; \Delta r \; 10^3}{3949 + 472\;8\left(\frac{1}{F} - 1\right)},$$

where V is the final volume of the gaseous system in liters. With V approximately the same (0 81 liters in the present experiments) and F assumed to vary only to a small extent, then approximately the CO_2 increment is proportional to Δr Furthermore the relation given in (1) is independent of temperature or pressure, and the humidity may be *fixed* arbitrarily

The results to be reported at present will be given in graphic form, using the *median age* of larvae (α) in days as abscissa, with $10^3 \; \Delta r$ per minute as ordinate—one tenth the directly observed Δy for a 20 minute interval—to indicate respiratory rate, and on a sub graph as in the previous work[4] an estimate of the fraction of the larvae having pupated

EXPERIMENTAL RESULTS

The results of an experiment where lots of larvae were irradiated for 1/4, 1/2, and 1 hour, respectively, at a median age approximately 3 5 days are presented in contrast to a control lot in Text fig 1 Although there appears to be little or no effect on the duration of the larval stage, the initial effects upon respiration closely resemble those obtained with x rays No great difference is apparent as a result of the variation of the duration of irradiation, but it may be assumed that such differences would appear if shorter irradiations were made That this is so and that the effect observed is due principally to the ultraviolet component of the radiations is indicated by another experiment where in two lots of larvae were similarly irradiated for 1/2 hour, except that one lot was protected by an interposed pane of clear glass (cleared x ray plate) which filtered out a considerable part of the ultraviolet radiation The results, including observations on a control lot, are given in Text fig 2

In a third experiment four lots of larvae were observed, three of which were given a single irradiation for 30 minutes as in the first experiment but at ages about 1 day apart The results are given in Text fig 3, where there appears to be a greater deviation immediately after irradiation the older the larvae, but less total displacement of the respiratory curve The initial depression of respiratory rate after
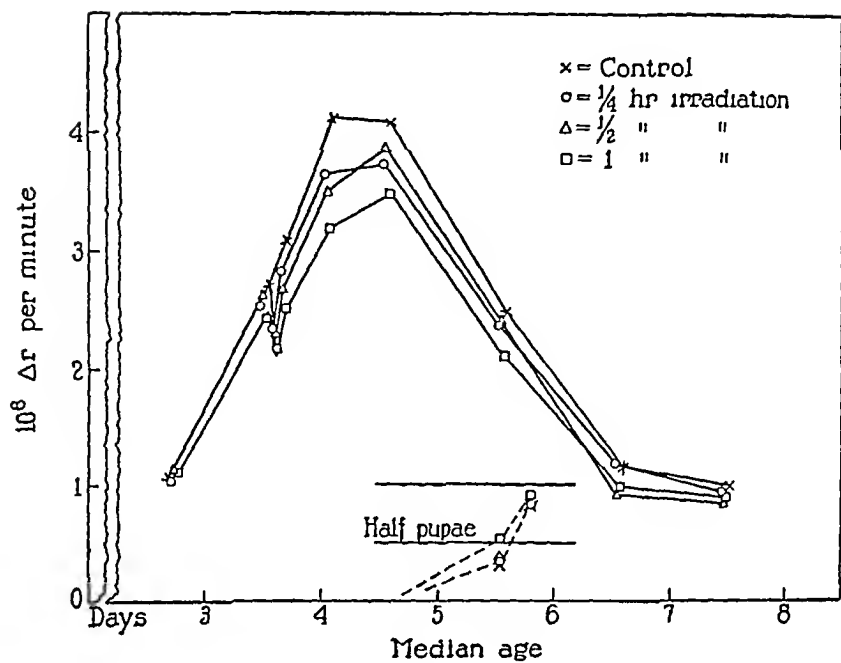
FIG 1
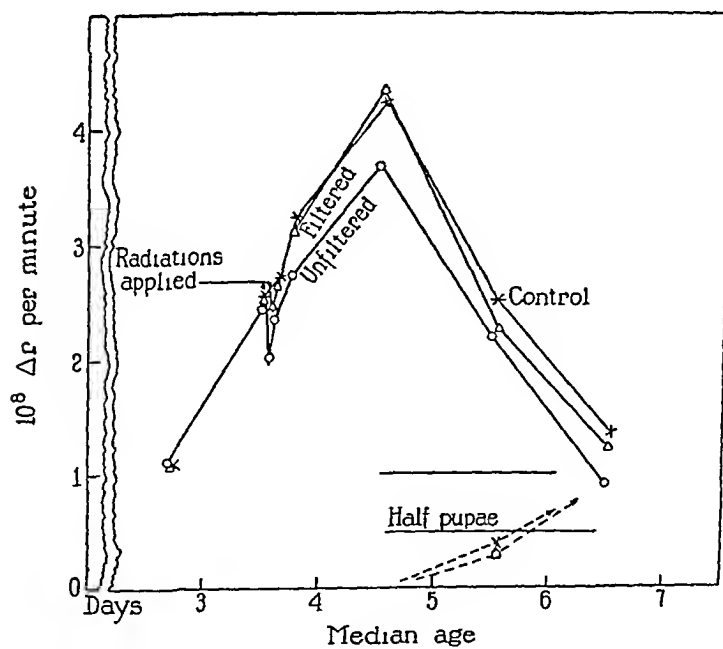
FIG 2
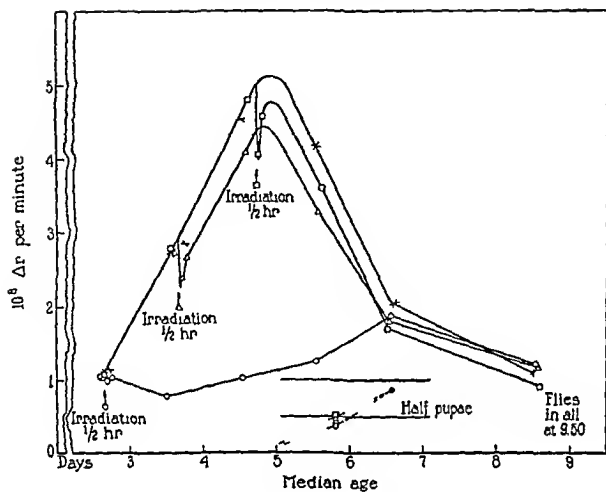
Fig 3
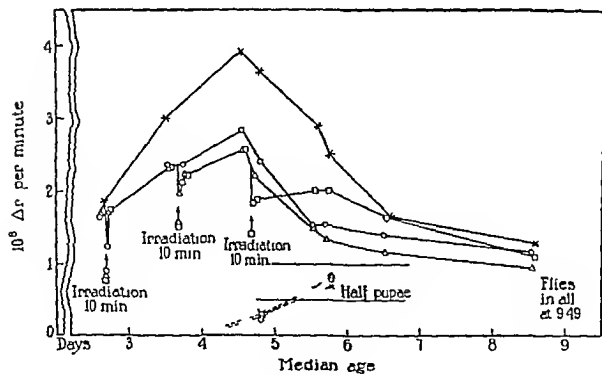
Fig 4

irradiation appears to be roughly proportional to the respiratory rate just before   Again the median age at pupation is sensibly the same in all lots, and imagos appeared in each lot at about the same time

Similarly with four lots, A, B, C, and D, an experiment was performed where D was the control, and all other lots were irradiated simultaneously on the 1st day, A and B again simultaneously on the 2nd day, and A once more on the 3rd day—all irradiations being of 10 minute duration, with the result represented in Text-fig 4   Time of pupation and of emergence of imagos again appear little affected, and the same depressing effect on CO_2 respiration appears after each irradiation

DISCUSSION

Over ranges wherein certain lots were still experimentally the same, the close comparability of the respective respiratory rates even to the extent of close agreement in response to a second irradiation in the last experiment is remarkable   The results given include all the observations made in four of the first seven experiments wherein respiration of larvae was used as an indicator of effects of ultraviolet irradiation The first and third experiments presented are the better of duplicate experiments indicating roughly the same results   Success in developing precise *mass* phenomena depends obviously on the technique of preparation and random sampling of the material

In further work[9] where the acetic acid concentration of the food differed in pairs of lots of larvae after their random selection from a common batch—one of each kept as a control and the other irradiated with ultraviolet light—there appeared a marked concomitant variation in sensitivity   Interesting in connection with this is a consistent decrease noted[7] in former work in sensitivity of larvae to x-ray irradiation (as evidenced by a halving of the extension of the prepupal period) when larvae sealed in paraffin-stearin wells were not provided with the usual ventilation during irradiation   Further study of the influence of pH and CO_2 tension upon sensitivity of biological systems to various forms of irradiation appears to offer prospects of interesting results   That such influence may be considerable, although of different significance in different situations, is indicated in the case of

mammals by the studies of Hussey,[11] [1] Sprunt,[13] and others on the influence of x ray irradiation on the properties of the blood

A considerable number of the observations in these studies were made by Mr Emery F Barringham

SUMMARY

1 Although ultraviolet irradiation of larvae did not prolong the larval stage as is the case with x rays, the immediate effects on respiratory rates are strikingly similar

2 Effects of irradiation at different ages were observed and also effects of successive irradiations with remarkable agreement in comparable lots

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[12] Hussey, R , *J Gen Physiol* , 1922–23, 5, 359

[13] Sprunt, D H , *J Biol Chem* , 1931, 92, 605

ELECTRIC IMPEDANCE OF HIPPONOË EGGS

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The results on the alternating current impedance of *Arbacia* eggs (Cole, 1928 b) indicated that a polarization type of impedance predominated at the surface of the egg This is also the case for many tissue membranes (Fricke, 1931, Cole, 1932, Bozler and Cole, 1935) whereas the impedance of the membranes of red blood cells, (Fricke, 1925) and yeast (Fricke and Curtis, 1934) is predominantly that of a static capacity Because of the similarity between the eggs and tissues and since the analysis of data can be much more complete for suspensions of spherical cells than for tissues, it was desirable to extend the previous work Measurements of the resistance and capacity should completely determine the polarization characteristics of the membrane as was not possible from the measurements of scalar impedance made on *Arbacia* eggs The work on muscle (Bozler and Cole, 1935) and *Laminaria* (with B M Hogg, unpublished) emphasized the importance of the zero frequency conductance of the membrane, but the measurements of the volume concentration of the *Arbacia* suspensions were not accurate enough to be used for this purpose

Preparation of Material

Some difficulty was experienced in obtaining a sufficient quantity of *Arbacia* eggs from a single female, so it was decided to work with *Hipponoë esculenta* one female of which yields as much as 100 cc of settled eggs between October and January in Bermuda

The urchins were either injured and allowed to shed the eggs into sea water or the ovaries were removed and placed in sea water The suspensions were strained through coarse bolting cloth It was found that with jelly present, the volume concentration of settled or lightly centrifuged eggs was often less than 20 per cent This jelly was difficult to remove entirely by shaking without injury to the eggs but a mild shaking removed part of it and gave 30 per cent to 50 per cent

concentrations of eggs in good condition   The rather heavy suspension was then placed in a large crystallizing dish with 2 or 3 liters of sea water and allowed to settle   The supernatant sea water and detached jelly were siphoned off and the eggs given another such washing   Before measurement, the suspension was cleared of jelly-free eggs and excess sea water by light centrifuging

Apparatus

Conductivity Cell —It was planned to use a bubbler cell of the type developed for the *Arbacia* eggs, but the *Hipponoe* eggs were in better condition after standing in jelly than after stripping and bubbling   The conductivity cell was made in the form of a burette, 0 64 cm inside diameter and 24 cm long, including the burette stop-cock at the lower end   Cylindrical electrodes, 0 5 cm long, were sealed into the wall of the cell, 9 3 cm apart   This cell could be conveniently filled by suction and delivered 5 08 cc from the fiducial mark above the upper electrode   The electrodes were platinized platinum and the cell constant was 29 5   When the suspensions were diluted with sea water and inseminated after an hour in the cell, normal fertilization and cleavage took place in practically all of the eggs   Within 10 or 15 minutes after the eggs were drawn into the cell, the resistance and capacity reached steady values which only changed slowly over a period of hours, if there were no appreciable temperature changes   Most of the measurements were made at temperatures between 21 and 24°C

Wheatstone Bridge —The parallel resistance and capacity measurements were made with the same alternating current Wheatstone bridge used for the muscle impedance (Bozler and Cole, 1935) at eleven frequencies from 1 08 kc (kilocycles per second) to 2 32 10^3 kc   The substitution method was employed at all frequencies   The egg cell and precision condenser in parallel were first balanced directly against another air condenser and electrolytic resistor (Bozler and Cole, 1935) in parallel on the other arm of the bridge   The egg cell was then replaced by a second electrolytic resistor and the bridge again balanced by adjustment of this resistor and the precision condenser

The resistance of the unknown was then found from calibrations of the electrolytic resistor made before and after each run   The capacity of the unknown was given by the sum of (1) the change in capacity of the precision condenser, (2) the capacity of the electrolytic resistor, and (3) the difference in capacities of the leads to the cell and to the resistor   The error due to changes of inductance was negligible   An entire frequency run could be made in less than 15 minutes

Measurements and Results

Volume Concentration and Membrane Resistance

The specific resistance, r, of a suspension of homogeneous spheres is given by the Maxwell equation

$$\frac{1 - r_1/r}{2 + r_1/r} = \rho\, \frac{1 - r_1/r_2}{2 + r_1/r_2} \tag{1}$$

where ρ is the volume concentration and r_1 and $\hat{r}_2$ are the specific resistances of the medium and the spheres respectively Since the suspended cells are not homogeneous they may more reasonably be assumed to consist of an electrically homogeneous interior of specific resistance, r_2, surrounded by a thin membrane having a parallel capacity C_0 and resistance r_3 per unit area When the radius of the sphere is a, it can be shown (Cole, 1928 a) that the specific resistance $\hat{r}_2$ of the equivalent homogeneous sphere is

$$r_2 = r_2 + r_3/a$$

At sufficiently low frequencies, the reactance of the membrane, $1/C_0\omega$, will be large compared with its resistance r_3 and the specific resistance of the suspension, r, will approach its limiting value r_0 If the membrane is non conducting, r_3, and consequently $\hat{r}$, is infinite so

$$\frac{1 - r_1/r_0}{2 + r_1/r_0} = \frac{\rho_0}{2}$$

where ρ_0 is the "non-conducting volume concentration," (Fricke and Curtis, 1935) It cannot in general be assumed that the membrane is a non conductor at low frequencies, so that an independent measure of ρ is necessary to determine $\hat{r}_2$ If it is then found that there is no significant difference between ρ and ρ_0 it may be concluded that, within the experimental error, the membrane is non-conducting and the cells are equivalent to non-conducting spheres

The volume concentrations of the *Arbacia* suspensions were calculated from the centrifuged volume of eggs The method seemed quite unreliable, as Gerard and Rubenstein (1934) have pointed out, and could not be used for *Hipponoe* eggs

The hemoglobin colorimetric method for the measurement of volume concentration has been very successful for blood (Ponder and Saslow, 1930) and preliminary experiments by Ponder at Cold Spring Harbor on *Asterias* eggs indicated that it might be generally applicable to marine eggs Unfortunately the hemoglobin seemed to be absorbed by the jelly in such large amounts that it could not be used, and the dye indigo carmine was then tried It does not penetrate A ^{1n}

eggs and is not appreciably absorbed by the *Hipponoe* jelly   A series of measurements all gave volume concentrations ρ considerably higher than the non-conducting concentration ρ_0 The calculated values of membrane resistances were quite low and not at all consistent with each other   From the capacity measurements, there was reason to suspect a high membrane resistance which gave additional cause to suspect the determinations   It seems probable that sufficient colloidal material, from the jelly, remained in the color sample from the suspension, to falsify the color match against the standard made up from fresh sea water
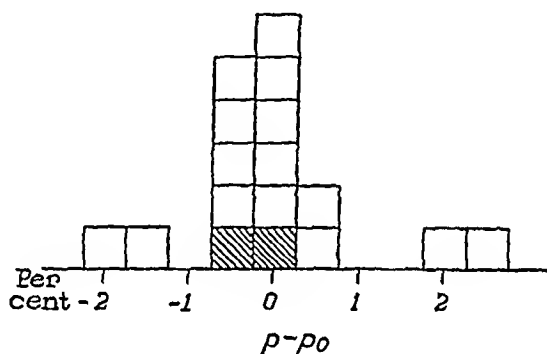
FIG 1 *Frequency distribution of the differences of the titration percentage volume concentration,* ρ, *and the low frequency non-conducting percentage volume concentration,* ρ_0, *for Hipponoe eggs   Open blocks for unfertilized suspensions, and shaded blocks for fertilized suspensions*

The next attempt was essentially a conductance titration   The egg suspension in the cell, 5 08 cc was run into 10 cc of iso-osmotic dextrose solution, the eggs removed by centrifuging, and the resistances of the supernatant solution and sea water measured   From their ratio, and a calibration curve, the volume of sea water added to the dextrose, and consequently the egg volume concentration of the original suspension, ρ, could be determined   At the same time, a standard containing the amount of sea water corresponding to the non-conducting volume concentration, ρ_0, was measured as a check

The frequency distribution of the differences, $\rho - \rho_0$ is shown in Fig 1   Two suspensions of fertilized eggs are included and two determinations have been omitted   In one of the latter some cytolysis

took place and in the other the eggs settled slightly in the conductivity cell and left clear sea water above, which was not removed

There is no evidence to indicate any injury or salt leakage of the eggs in the dextrose solution   The surface capacity and internal resistance of the eggs were not affected and there are no systematic differences between ρ and ρ_0 The eggs with their attached jelly probably assumed a somewhat regular arrangement in the cell, but this did not seem to invalidate the application of the Maxwell equation It may be concluded that within the limits of experimental error, the cell membranes of both the unfertilized and fertilized *Hipponoe* eggs are non conducting
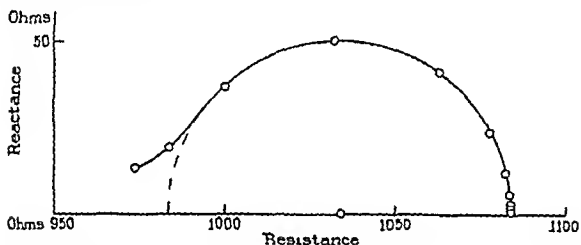
FIG 2 Resistance R vs reactance λ in ohms for a 39 3 per cent suspension of unfertilized *Hipponoe* eggs

Membrane Capacity

The equivalent series resistance, R, and reactance, λ, were computed for each frequency, n, from the observed parallel resistance, R_p, and capacity, C_p, by the formulae,

$$R = R_p \quad \lambda = R_p^2 C_p \omega$$

where $\omega = 2\pi n$ and the $R_p C_p \omega^2$ term is negligible   The complex plane locus (Cole, 1928 a) for an unfertilized suspension is shown in Fig 2 It is seen that the phase angle of the variable impedance element is $90°$ over most of the frequency range, so it is concluded that the membrane impedance is predominantly that of a static capacity   This agrees with the observations at frequencies from 1 08 kc to 10 8 kc

that the parallel resistance and capacity of all suspensions were constant within the limits set by temperature variations and electrode polarization errors

The membrane capacity per unit area, C_0, may then be computed from the formula (Cole, 1928 a)

$$C_0 = \frac{2c\,C_p}{(2 + r_1/r_0)\,(1 - r_1/r_0)\,a}$$

where c is the cell constant, C_p the parallel capacity of the suspension, r_1, and r_0, the resistances of sea water and the suspension at low frequency, respectively, and a the egg radius   For the suspension of Fig 2, $c = 29\ 5$, $C_p = 70\ \mu\mu f$, $r_1 = 552$ ohms, $r_0 = 1082$ ohms, and $a = 39\ 5\mu$, so $C_0 = 0\ 85\ \mu f/cm^2$ After standing several hours, the membrane capacity decreased, occasionally as much as 10 per cent   The
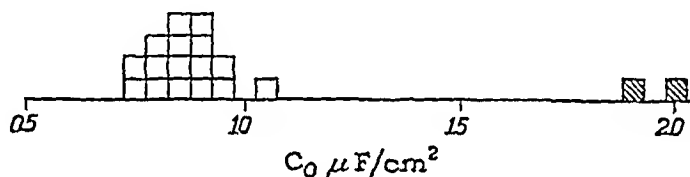
FIG 3   Frequency distribution of the membrane capacity C_0 of *Hipponoë* eggs Open blocks for unfertilized eggs and shaded blocks for fertilized eggs

frequency distribution of the first measurement of C_0 on the eggs of sixteen females is shown in Fig 3

For unfertilized eggs, the average $C_0 = 0\ 87\mu f/cm^2$, which is close to the value of $0\ 81\ \mu f/cm^2$ for the red blood cell (Fricke, 1925) and $0\ 6\mu f/cm^2$ for yeast (Fricke and Curtis, 1934)   It is to be noticed that there is a rather wide variation between the eggs of different urchins, the largest value being $1\ 07\mu f/cm^2$ and the two smallest, $0\ 77\mu f/cm^2$

Quite by chance, the eggs of the two latter urchins were chosen for fertilized experiments   After insemination, less than 1 per cent of the eggs failed to raise membranes and nearly all underwent normal third cleavage   No measurements were made less than 10 minutes after insemination, but at that time one had a membrane capacity of $1\ 9$ $\mu f/cm^2$ and the other $2\ 0\ \mu f/cm^2$ or an average of two and a half times the capacity of the unfertilized eggs   No changes were observed during the half hour the eggs were in the cell

Internal Specific Resistance

The departure of the high frequency points from the semicircle is believed to be real, although it is just at the limit of the experimental error   Consequently an extrapolation to the infinite frequency resistance is difficult to justify   However, if the measurements are not in error, they strongly suggest the entrance of another reactive element into the picture   The divergence is more marked and extends to lower frequencies in the complex plane locus of the fertilized eggs shown in Fig 4 which lends considerable weight to the hypothesis From this viewpoint, the internal resistance, r_2, calculated by Equation 1 from $r = r_\infty$, the high frequency intercept of the semicircle on
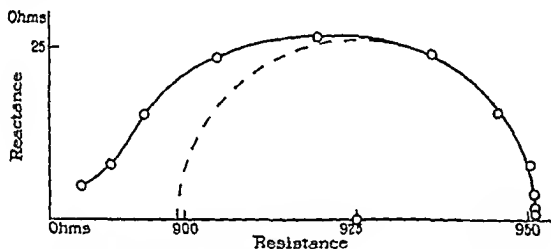
F1G 4, Resistance R vs reactance λ in ohms for a 29 7 per cent suspension of fertilized *Hipponoë* eggs

the resistance axis, should be the equivalent low frequency resistance of the egg interior   Then the values of r_2/r_1 range from 9 5 to 13 7 The average low frequency internal specific resistance is eleven times that of sea water for the unfertilized egg and eighteen times for the fertilized egg

Swelling Experiments

Low frequency measurements were made on two lots of unfertilized eggs in equilibrium with successive dilutions of sea water   The eggs were measured in 100 per cent sea water and then in 80 per cent, 60 per cent, and 40 per cent sea water with a half hour allowed for them to reach equilibrium after each change   The egg diameters were not

measured, but were computed on the basis of an 11 per cent osmotically inactive volume as found for *Arbacia* (McCutcheon, Lucké, and Hartline, 1931)   The membrane capacity per cm ^{2} *vs* per cent increase of surface area is plotted in Fig 5 for one series   It is very interesting that the specific capacity should be a linear function of the surface   Since the capacity per unit area increases as a macroscopic dielectric is made thinner, the observed decrease is surprising and puzzling, and suggests a constitutional change in the membrane   A single high frequency run in 44 5 per cent sea water showed a 37 per cent increase of the equivalent internal specific resistance
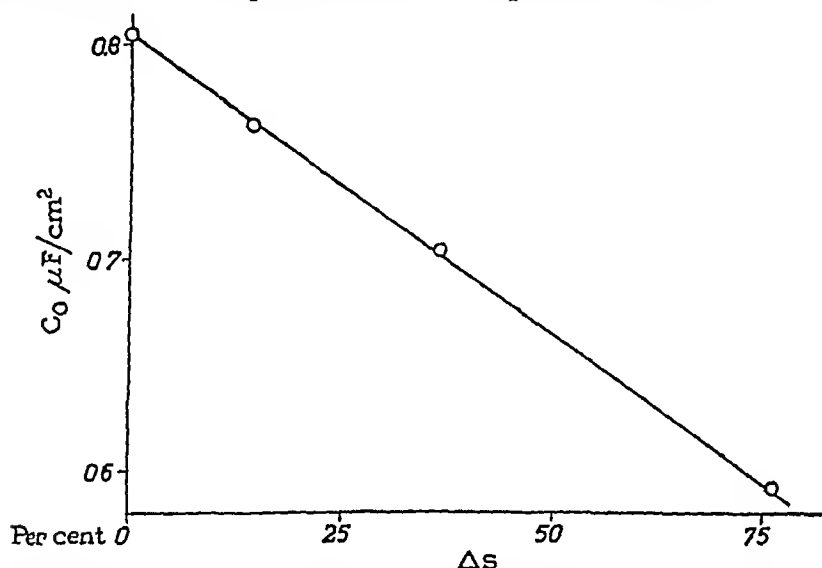
FIG 5   Increase of surface area ΔS in per cent of normal *vs* C_0 capacity per cm ^{2} of egg membrane for swollen unfertilized *Hipponoe* eggs

DISCUSSION

The reactance of many tissues and of *Arbacia* eggs seems to be largely due to a membrane impedance of the type $z(\omega) = z_1(j\omega)^{-\alpha}$ (Cole, 1934) found in electrolytic electrode polarization and the selenium barrier layer photocell   Although there is no adequate theoretical basis for these phenomena or the empirical equation, the effect may be qualitatively explained as due to a back electromotive force resulting from a selective transfer of ions across the surface of discontinuity   For zero permeability of all ions, $\alpha = 1\ 0$, for complete

permeability, $\alpha = 0$, and for perfect permeability for one ion and zero for the other ion of a binary electrolyte, $\alpha = 0.5$ on the Warburg (1899) theory Thus for the case $\alpha < 1.0$, some ionic permeability is to be expected If then there is any permeability, we should expect a finite membrane resistance at zero frequency, and if there is a selective permeability we should expect α to be greater than zero and less than unity

Thus it has been difficult to understand the relation of the ion permeability postulated from other considerations to the observed high resistance and static capacity of the red blood cell membrane However, the experiments of Fricke and Curtis on blood (1935) and yeast (1934) suggest that the static capacity which predominates at high frequencies is of relatively slight physiological importance and that it is the small increments of resistance and capacity at low frequencies which seem to have the characteristics of a polarization impedance and may be correlated with the physiological condition of the cells On the other hand the increase of the static capacity of *Hipponoe* eggs on fertilization and the decrease on swelling indicate that the seat of this capacity is not entirely inert When both a static capacity and a polarization impedance are present, the former may predominate when the membrane is relatively impermeable and the latter when the permeability is greater To predict the frequency range for the appearance of each would require a justifiable equivalent membrane circuit

There is no obvious reason to suspect a large difference in the ion permeabilities of the *Hipponoe* and *Arbacia* eggs so it was quite surprising to find the predominance of the static capacity in the former when the latter indicated a polarization impedance As careful measurements as the conditions would permit down to 1.08 kc gave no definite evidence of the existence of a polarization impedance for the *Hipponoe* eggs Even if present it might be very difficult to detect because of the low resistance of sea water and the high internal resistance of the eggs

The internal specific resistance of eleven times that of sea water seems singularly high since *Arbacia* gave 3.5 times sea water, red blood cells twice plasma, and frog sartorius 3.5 times Ringer If the nuclear membrane enters at the high frequencies, then it would be effectively

non-conducting at the frequencies for which this resistance is calcu-
lated   If the cytoplasm has a specific resistance 3 5 times that of sea
water then from the Maxwell equation for a two phase sphere (Cole,
1928 *a*) the nucleus would occupy 60 per cent of the unfertilized egg
and 73 per cent of the fertilized egg   Again, the nuclear membrane
may not be involved and the internal resistance may be low, but the
high frequency divergence due to a polarization impedance at the
plasma membrane in such relation to the static capacity that it appears
at high frequency rather than low   It is not possible at present to say
whether the pronounced effect in the fertilized egg is due to a change
in the postulated unknown element or whether it is the same as in the
unfertilized egg, but unmasked by the increase in the static capacity on
fertilization

The author is particularly indebted to Dr H J Curtis of the Bio-
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eration in this work

SUMMARY

Alternating current resistance and capacity measurements have
been made from 1 08 10³ to 2 32 10⁵ cycles per second on suspensions of
unfertilized, fertilized, and swollen unfertilized eggs of the echinoderm
Hipponoe esculenta   A simple method has been developed for measur-
ing the volume concentration of eggs in a suspension
The membrane of the unfertilized egg is practically non-conducting
at low frequencies and shows a static capacity of 0 87 $\mu f/cm^2$ except
perhaps at the highest frequencies   The equivalent specific resistance
of the egg interior is 11 times that of sea water
The membrane of the fertilized egg is practically non-conducting at
low frequencies and shows a static capacity 2 5 times that of the
unfertilized egg except at the higher frequencies where another reactive
element produces a marked effect   The internal resistance is appar-
ently higher than that of the unfertilized egg
The static capacity per unit area of the membrane decreases as a
linear function of the surface area when the eggs are swollen in dilute
sea water   In 40 per cent sea water, the capacity falls to about 75 per
cent of normal

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THE SOLUBILITIES, APPARENT DISSOCIATION CONSTANTS, AND THERMODYNAMIC DATA OF THE DIHALOGENATED TYROSINE COMPOUNDS

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The present work is a continuation of the systematic survey of the solubilities, apparent dissociation constants, and thermodynamic properties of the amino acids, which for some time have been the subject of study in this laboratory (1) We are reporting the solubility in water of d tyrosine, dl tyrosine, diiodo dl tyrosine, dibromo l-tyrosine, and dichloro l tyrosine over a temperature range of from 0° to 50°C , and the apparent dissociation constants of dibromo l tyrosine and dichloro l tyrosine at 25° and 40°C From these data we have calculated the heats of solution of all of the above amino acids and the apparent heats of ionization of dibromo l-tyrosine and dichloro l-tyrosine

Methods

The technique employed for obtaining the solubilities of the amino acids was the same as described by Dalton and Schmidt (2) The concentrations of the amino acids in the saturated solutions were obtained by micro Kjeldahl determinations of nitrogen (3) The criteria of purity of the preparations were constant solubility after repeated recrystallization and constant solubility when there was a small excess and a large excess of the solid phase

For estimating the apparent dissociation constants, the solubilities of the amino acids were determined in aqueous solutions and in solutions containing varying amounts of sodium hydroxide or hydrochloric acid The hydrogen ion activities of the solutions were determined with the aid of the quinhydrone electrode They are based on the value 1 078 for the paH of 0 1000 molar HCl (4), and on the assumption that contact potential differences were eliminated by the use of saturated potassium chloride It was found impossible to use a hydrogen electrode because dibromo-l tyrosine and dichloro-l tyrosine are easily reduced to tyrosine by hydrogen in the presence of platinum black This procedure for

estimating the apparent dissociation constants of amino acids having a low solubility has been used by Hitchcock for *l*-tyrosine (5) and by Dalton, Kirk, and Schmidt for diiodo-*l*-tyrosine (6)   The present work completes the data on the apparent dissociation constants for the dihalogen substitution products of *l*-tyrosine which, up to the present time, have been found to occur naturally or which have been synthesized (7, 8)

<h2 style="text-align:center">EXPERIMENTAL</h2>

l-Tyrosine was obtained by enzymatic hydrolysis of casein   After recrystallizing it several times from hot water, the specific rotation of a 4 8 per cent solution in 1 0 molar HCl was found to be $(\alpha)_{5461}^{20} = -12\,4$ Analyses gave theoretical values for nitrogen   Its solubility at 25° was within 0 5 per cent of the value given by Dalton and Schmidt (2)

dl-Tyrosine was prepared in two ways   *l*-Tyrosine, obtained by the above method, was boiled 24 hours with 10 times its weight of 40 per cent sodium hydroxide solution   The racemic tyrosine was precipitated with 50 per cent acetic acid and recrystallized several times from hot water   *dl*-Tyrosine was also synthesized by a method furnished us by Professor M S Dunn of the University of California at Los Angeles [1]   The main steps in the synthesis were as follows   malonic ethyl ester → bromomalonic ethyl ester → phthalimidomalonic ethyl ester → sodium phthalimidomalonic ethyl ester → *p*-methoxybenzyl phthalimidomalonic ethyl ester → *dl*-tyrosine hydrobromide → *dl*-tyrosine   Both preparations of *dl*-tyrosine on analysis gave theoretical values for nitrogen and possessed no optical activity   Their solubilities were within 1 5 per cent of each other   Twenty-four solubility measurements were carried out at eight different temperatures between 273 1° and 332 6° absolute

In Table I equations which express the solubility relationships as a function of the temperature are given for *dl*-tyrosine and the other amino acids studied   The equations were obtained by the method of least squares   In Table II the solubilities of the amino acids have been calculated on the basis of these equations in grams per 1000 gm of water   The thermodynamic relationship between ΔH, the differential heat of solution, N_2, the mol fraction, T, the temperature, and P, the pressure, can be expressed as follows (13)

$$\left(\frac{\partial \ln N_2}{\partial T}\right)_P = \frac{\Delta H}{RT^2} \tag{1}$$

ΔH is also the total heat of solution of 1 mol of solute in an infinite amount of saturated solution   In dilute solutions, such as we have

---

[1] Private communication

TABLE I

Coefficients of Solubility Equations* of Tyrosine and the Halogen Substitution Products of Tyrosine

Substance	a_1	$b_1 \times 10^3$	$c_1 \times 10^4$	a_2	a_3	$b_3 \times 10^3$	$c_3 \times 10^4$	a_4	$b_4 \times 10^3$	$c_4 \times 10^4$	Maximum deviation	Mean deviation
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)		
l Tyrosine†	−0 708	1 46		−2 966	−10 799	3 36		−20 062	3 37		+3 40	±1 43
d Tyrosine	−0 703	1 46		−2 966	−10 799	3 36		−20 062	3 37		+3 40	±1 43
dl Tyrosine	−0 833	1 51		−3 091	−16 562	3 46		−20 577	3 46		+3 00	±1 73
Diiodo-l tyrosine†	−0 690	1 92		−3 326	−19 745	4 42		−23 761	4 43		−5 18	±1 15
Diiodo-dl tyrosine	−0 827	1 43		−3 464	−16 989	3 90		−21 006	3 30		−5 58	±2 54
Dibromo-l tyrosine (hydrate)	0 0839	1 627		−2 445	−15 881	3 753		−19 894	3 752		+2 15	±1 06
Dibromo-l tyrosine (anhydrous)	0 183	0 9984		−2 343	−11 610	2 246		−15 537	2 247		−1 00	±0 71
Dichloro-l tyrosine	0 0065	1 038	4 648	−2 392	−4 058	−3 450	1 069	−8 426	−3 215	1 030	−1 34	±0 62

* Solubility equations

$$\log S = a_1 + b_1 t + c_1 t^2 \qquad (1)$$
$$\log m = a_2 + b_2 t + c_2 t^2 \qquad (2)$$
$$\ln m = a_3 + b_3 T + c_3 T^2 \qquad (3)$$
$$\ln N_2 = a_4 + b_4 T + c_4 T^2 \qquad (4)$$

The constants for Equation (1) are given in Columns 2 to 4   The constants for Equation (2) are given in Columns 3 to 5
The constants for Equation (3) are given in Columns 6 to 8   The constants for Equation (4) are given in Columns 9 to 11
† The values for l tyrosine and diiodo l tyrosine are taken from the paper by Dalton and Schmidt (2)

used, the value for ΔH can be assumed to represent the total heat of solution Since $\ln N_2 = 3\,46 \times 10^{-2}T - 20\,577$, $\Delta H = 6\,88 \times$

TABLE II

Table of Calculated Solubilities of Tyrosine and the Halogen Substitution Products of Tyrosine

t	l-Tyrosine	d Tyrosine	dl Tyrosine	Diiodo l tyrosine	Diiodo-dl tyrosine	Dibromo-l tyrosine (hydrated)	Dibromo l-tyrosine (anhydrous)	Dichloro-l tyrosine
°C								
0	0 196	0 196	0 147	0 204	0 149	1 213		1 015
5	0 232	0 232	0 175	0 255	0 176	1 463		1 147
10	0 274	0 274	0 208	0 318	0 207	1 764		1 303
15	0 324	0 324	0 248	0 396	0 244	2 128		1 488
20	0 384	0 384	0 294	0 494	0 288	2 566	2 432	1 709
25	0 453	0 453	0 351	0 617	0 340	3 095	2 723	1 973
30	0 537	0 537	0 417	0 769	0 400		3 055	2 289
35	0 635	0 635	0 497	0 959	0 472		3 420	2 671
40	0 752	0 752	0 590	1 197	0 556		3 828	3 134
45	0 889	0 889	0 703	1 493	0 656		4 295	3 696
50	1 052	1 052	0 836	1 862	0 773		4 808	4 382

These values were calculated on the basis of equations given in Table I   All values are in terms of grams per 1000 gm of water

TABLE III

Differential Heats of Solution of Certain Amino Acids

Amino acid	Differential heat of solution
	cal
l-Tyrosine	5,960
d-Tyrosine	5,960
dl-Tyrosine	6,110
Diiodo-l-tyrosine	7,830
Diiodo-dl-tyrosine	5,830
Dibromo-l-tyrosine (anhydrous)	3,970
Dibromo-l-tyrosine (hydrated)	6,630
Dichloro-l-tyrosine	5,170

$10^{-2}T^2$ At 298 1° absolute, the value of ΔH for dl-tyrosine is 6,110 calories   In Table III the differential heats of solution at 298 1° absolute of the amino acids studied are given

d Tyrosine was obtained by resolution of dl tyrosine using the method of fractional crystallization of the brucine salts of formyl dl tyrosine (9) The specific rotation of a 4 8 per cent solution in 1 0 molar HCl was found to be $(\alpha)_{MR}^{25^\circ}$ = +12 5 Analysis gave theoretical values for nitrogen Its solubility at three different temperatures was within 1 0 per cent of the value for l tyrosine given by Dalton and Schmidt (2)

The fact that the solubility of the racemic isomer is lower than that of the active forms is an indication that dl tyrosine is a racemic compound and not a mixture Further evidence for this was provided by the observation that l-tyrosine dissolves in a saturated solution of the racemic isomer If the latter had been a mixture this solution would

TABLE IV

Change of Solubility of a Mixture of d Tyrosine and l Tyrosine with Time

Time	Solubility
hrs	*gm pe 1000 gm H₂O*
1	0 951
3	0 957
6	0 932
12	0 914
24	0 899
48	0 892
72	0 380
120	0 355
144	0 353

have been saturated with respect to the levo isomer and the l tyrosine would not have dissolved when brought in contact with the saturated solution of dl tyrosine

The compound, dl tyrosine, is formed when d tyrosine and l tyrosine are mixed in aqueous solution This was demonstrated by the following experiment 75 mg samples of both d and l tyrosine were added to 100 cc of water at 25° The amount of tyrosine dissolved was determined in aliquots removed from time to time The results are given in Table IV

The solubility of the mixture at the end of 144 hours is within 1 0 per cent of the solubility determined for dl tyrosine Loring and du Vigneaud obtained similar results with a mixture of d and l-cystine

(10)   It is important that exactly the same amounts of *d*-tyrosine and *l*-tyrosine are used at the beginning of the experiment   If there is an excess of one form it will be left after the *dl*-tyrosine compound has been formed and the amount of tyrosine in solution will be the sum of the racemic compound plus the optically active form

The solubility, apparent dissociation constants, and thermodynamic data for diiodo-*l*-tyrosine have been reported by Dalton, Kirk, and Schmidt (6)   Oswald (11) had reported previously a solubility value for diiodo-*l*-tyrosine which is between 7 and 8 times greater than the value reported by these workers, and a solubility value for diiodo-*dl*-tyrosine that approximated the solubility found in this laboratory for the levo isomer   This suggested that, in the conversion of *l*-tyrosine into diiodotyrosine, racemization had taken place   To clear up this point, a sample of the diiodotyrosine used by Dalton, Kirk, and Schmidt was reduced to tyrosine by the procedure outlined by Haring-ton (12), using palladium black as a catalyst   The tyrosine obtained had the same optical activity as the *l*-tyrosine used in the preparation of the diiodotyrosine

Diiodo-*l*-tyrosine and diiodo-*dl*-tyrosine were prepared according to the method of Oswald (11), starting with *l*-tyrosine and *dl*-tyrosine prepared as described previously in this paper   The products on analysis gave theoretical values for iodine and nitrogen   The solubility of the diiodo-*l*-tyrosine was within 2 0 per cent of the value given by Dalton, Kirk, and Schmidt   The solubility of the diiodo-*dl*-tyrosine was lower than that of the levo isomer   Nineteen solubility measurements of the diiodo-*dl*-tyrosine were carried out at six different temperatures between 273 1° and 320 6° absolute   The usual equations were devised and their coefficients tabulated in Table I   By assuming that the perfect solution laws were obeyed, $\Delta H_{298\ 1}$, the differential heat of solution, was calculated and is given in Table III

Dibromo-*l*-tyrosine and dichloro-*l*-tyrosine were prepared by treat-ing *l*-tyrosine with bromine or chlorine in accordance with the pro-cedure recommended by Zeynek (8)   The synthesized compounds were reduced to tyrosine by the procedure outlined by Harington (12), using palladium black as a catalyst   No essential change in optical activity of the tyrosine was noted

FIG 1 Anhydrous form of dibromo *l* tyrosine
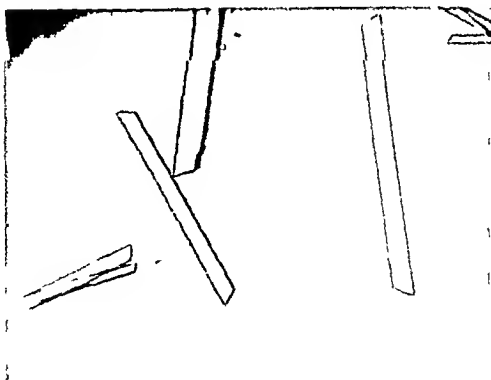
FIG 2 Hydrated form of dibromo *l* tyrosine

Dibromo-*l*-tyrosine crystallizes from water in two forms depending on the conditions under which the crystallization is carried out   When a boiling, saturated, aqueous solution is allowed to cool with no agitation, the compound crystallizes in long needles resembling salicylic acid and containing no water of crystallization (Fig 1)   After air drying, analysis gave, within experimental limits, theoretical values for nitrogen and bromine   When a warm solution is cooled in an ice bath with agitation, the compound crystallizes in platelets containing one half molecule of water of crystallization (Fig 2)   The interesting thing about these two forms
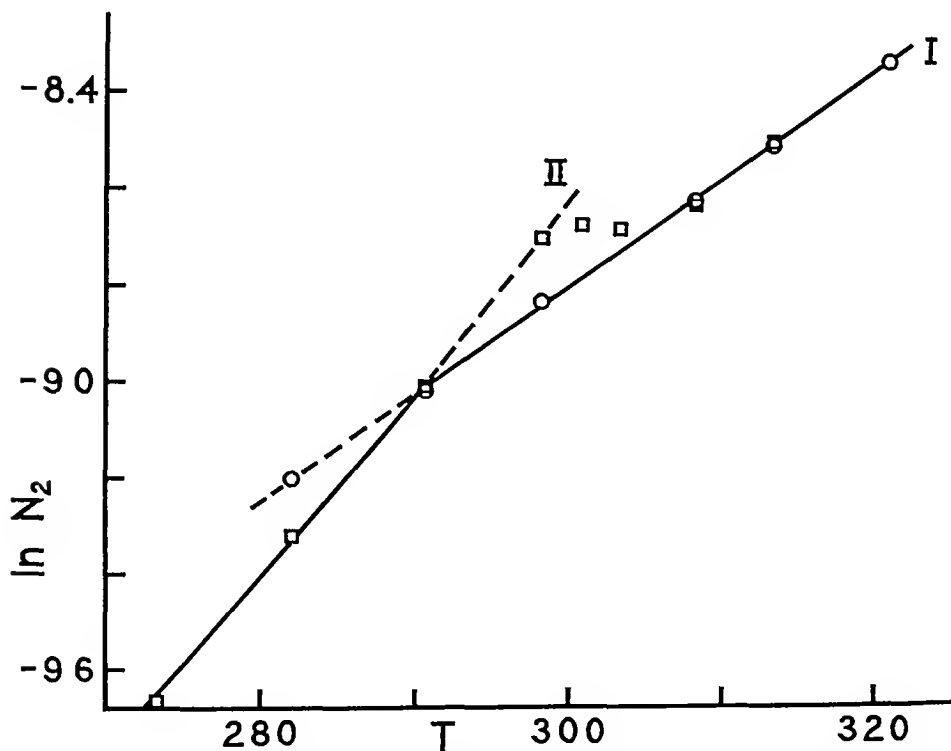
FIG 3 Solubility curves for anhydrous (Curve I) and hydrated (Curve II) dibromo-*l*-tyrosine

of dibromo-*l*-tyrosine is the difference in their solubility-temperature relationship   At 290 6° absolute, their solubilities are the same   At lower temperatures the hydrated form is the more stable, and at 273 1° absolute, the anhydrous form will change to the hydrated form if allowed to stand at this temperature for a sufficient length of time   Between 282 1° and 298 1° absolute, both forms are stable for the period of time investigated (up to 3 weeks), and exhibit differences in solubility   Above 306 1° absolute, the hydrated form will change to the anhydrous form if allowed to stand at this temperature for a sufficient length of time

Eleven solubility measurements were carried out on the hydrated form of dibromo *l* tyrosine at four different temperatures between 273 1° and 298 1° absolute   At higher temperatures the compound changed into the anhydrous form   The solubility relationships of the hydrated and anhydrous forms of dibromo *l* tyrosine are graphically represented in Fig 3   The usual equations were devised and their coefficients tabulated in Table I   The differential heat of solution was calculated and is given in Table III   Fourteen solubility measure
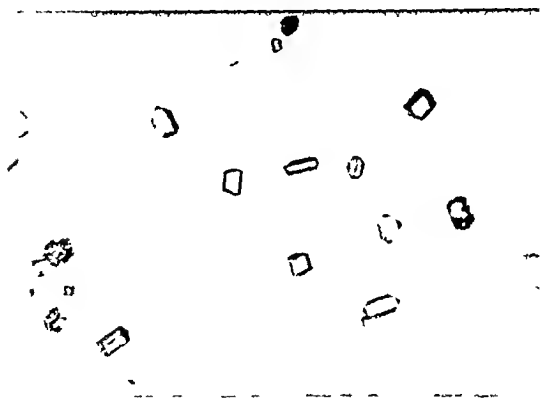
FIG 4 Dichloro *l* tyrosine

ments were carried out on the anhydrous form of dibromo *l* tyrosine at six different temperatures between 282 1° and 320 6° absolute   At higher temperatures the compound decomposed   The coefficients for the solubility equations are tabulated in Table I and the differential heat of solution is listed in Table III

Dichloro *l* tyrosine crystallizes from a hot aqueous solution at first as platelets which grow into prisms containing water of crystallization   An excellent description of the crystal form is given by Zeynek (8)   A photograph of these crystals is shown in Fig 4   Analysis of the preparation after drying in a vacuum desiccator

over phosphoric anhydride showed one molecule of water of crystallization Within experimental limits, theoretical values for nitrogen and chlorine were obtained

Fifteen solubility measurements in water on dichloro-l-tyrosine were carried out at seven different temperatures between 273 1° and 320 6° absolute   At higher temperatures the compound had a tendency to decompose   The usual equations expressing its solubility as a function of the temperature were devised and their coefficients tabulated in Table I   The differential heat of solution is listed in Table III

TABLE V

Apparent Dissociation Constants, Heats of Ionization, and Isoelectric Points of l-Tyrosine and Its Dihalogen Substitution Products

Substance	Temperature	pK'$_1$	pK'	pK'$_3$	pI
	°C				
l-Tyrosine	25	2 20	9 11	10 07	5 6
Diiodo-l-tyrosine	0	2 21	6 53	8 31	4 37
	25	2 12	6 48	7 82	4 29
	40	2 11	6 45	7 45	4 19
ΔH of ionization (calories)		980	810	8,790	
Dibromo-l-tyrosine (anhydrous form)	25	2 17	6 45	7 60	4 30
	40	2 11	6 41	7 28	4 23
ΔH of ionization (calories)		1,700	860	9,120	
Dibromo l-tyrosine (hydrated form)	25	2 16	6 44	7 58	4 28
Dichloro-l-tyrosine (hydrated form)	25	2 12	6 47	7 62	4 28
	40	2 08	6 42	7 31	4 22
ΔH of ionization (calories)		1,140	1,420	8,830	

For calculating the apparent dissociation constants of dibromo-l-tyrosine and dichloro-l-tyrosine from the solubility-paH data, the equations developed by Hitchcock (5) were employed   The data are presented in Table V   Because of the uncertainty as to which dissociation constant represents the ammonium ion and which represents the hydroxyphenyl group, the apparent dissociation constants, found in this investigation, are reported as K_1', K_2', and K_3', where $K_1' = \dfrac{K_w}{K_b'}$, $K_2' = \dfrac{K_w}{K_{a_1}'}$, and $K_3' = K_{a_2}'$ It is to be pointed out that very small errors either in the value obtained for K_2' or for (H$^+$) will make large

differences in the values of K_2' Because of this, the values obtained for K_3' indicate only roughly the order of magnitude of the dissociation constant

The question concerning the relative strengths of the ammonium ion and the hydroxyphenyl group in l tyrosine is a difficult one to decide Simms (14) assumed that the hydroxyphenyl group is the more acidic On the other hand, Cohn (15) considers that the ammonium ion, under the influence of the hydroxyphenyl group, is the more acidic and that it has about the same strength as the ammonium ions in phenylalanine and serine Birch and Harris (16) titrated l tyrosine in the presence of formaldehyde and from the displacement of the titration curve, came to the conclusion that the ammonium ion is the more acidic However, their work is open to question, because they did not consider possible reactions of the formaldehyde with the hydroxyphenyl group The displacement of the titration curve would probably be the same as they found if the hydroxyphenyl group were the more acidic

The measurements were carried out at 298 1° and 313 1° absolute for dichloro l tyrosine and for the anhydrous form of dibromo l tyrosine Measurements on the hydrated form of dibromo l tyrosine could be carried out only at 298 1° absolute, because at the higher temperature it changed to the anhydrous form For the purpose of calculating the apparent heats of ionization of these compounds, the equation of van't Hoff was used (13, p 298),

$$\frac{d \ln K}{dT} = \frac{\Delta H}{RT^2}$$

or

$$- \Delta H = \frac{d(R \ln K)}{d\left(\frac{1}{T}\right)} \tag{2}$$

where T = the absolute temperature, R = the gas constant in calories per degree, K = the true dissociation constant, and ΔH = the heat of ionization Since the true dissociation constants of these amino acids are not known, the assumption was made that the equation holds

when the values for the apparent dissociation constants are used instead of the true dissociation constants   The further assumption was made that ΔH is constant over the temperature range from 298 1° to 313 1° absolute, and, therefore, for this temperature interval, equation (2) may be written

$$-\Delta H = \frac{d(R \ln K)}{d\left(\dfrac{1}{T}\right)} = 28{,}490 \; \Delta \, \mathrm{p}K' \tag{3}$$

The dissociation values, heats of ionization, and isoelectric points of dibromo-l-tyrosine and dichloro-l-tyrosine are given in Table V   For comparison, the data of Hitchcock (5) relating to l-tyrosine and those of Dalton, Kirk, and Schmidt (6) concerning diiodo-l-tyrosine are included   From examination of the data given in Table V, certain conclusions can be reached l-Tyrosine and its dihalogenated substitution products have values of the same order of magnitude for $\mathrm{p}K_1'$ From the point of view of the zwitter ion theory this constant represents the dissociation of the carboxyl group and indicates that the substitution of the halogens in the hydroxyphenyl ring of l-tyrosine has very little effect on the dissociation of this group   The $\mathrm{p}K_2'$ and $\mathrm{p}K_3'$ values of l-tyrosine are much larger than in the case of the dihalogenated substitution products   This indicates that the groups represented by K_2' and K_3' are much more acidic in the case of the dihalogenated compounds than in l-tyrosine   Because of the increase in the acidic properties of these groups brought about by the introduction of the halogens in the hydroxyphenyl ring, the dihalogenated substitution products of l-tyrosine are stronger acids than l-tyrosine Since this increase in acidity is of the same order of magnitude, it appears that iodine, bromine, and chlorine, when substituted respectively to form the dihalogenated tyrosine compounds, have about the same effect on the dissociation constants

A general rule in organic chemistry, usually valid, is that the substitution of a strongly negative group, such as the halogens, will increase the acidic properties of groups in the molecule which have a tendency to give off hydrogen ions   This effect is greater the closer the negative group is to the group dissociating to give hydrogen ions If this rule is assumed to hold true, it would be expected that the

introduction of halogens adjacent to the hydroxyl group in the hydroxyphenyl ring of l tyrosine would increase the acidity of this group much more than that of the ammonium ion which is separated from the halogens by several carbon atoms   From the values for pK ' and pK_2' of l tyrosine and its dihalogenated substitution products, it must follow that pK ' represents the dissociation of the hydroxyphenyl group and pK_3' represents the dissociation of the ammonium ion in the dihalogenated substitution products of l-tyrosine   This conclusion is independent of which group is more acidic in l tyrosine

It has been pointed out by Kolthoff (17), Ebert (18), and Meyerhof (19), among others, that strongly acid groups have very small heats of ionization, whereas strongly basic groups possess large heat values—in the neighborhood of 10,000 to 12,000 calories   Therefore, it should be possible to predict the nature of the group which is dissociating by estimating the magnitude of the apparent heat of ionization   The organic radicals which are either weakly acidic or basic, such as the hydroxyphenyl and the imidazole rings respectively, will yield intermediate values for the heats of ionization and by this criterion should be readily identified

It might be expected from the apparent heats of ionization of the dihalogenated substitution products of l tyrosine that the groups could be identified which give rise to the three dissociation constants K_1' represents the dissociation of the carboxyl group and the heats of ionization, calculated from its values at different temperatures in the case of diiodo l tyrosine, dibromo l tyrosine, and dichloro l tyrosine, are small as shown in Table V   This is to be expected for a strongly acidic group

The values for the heats of ionization of the groups represented by K ' and K_2' are of no use in distinguishing between the ammonium ion and the hydroxyphenyl group in the dihalogenated substitution products of l-tyrosine, because sufficient data are not available for purposes of determining the heats of ionization that would be expected at the hydrogen ion activities at which they dissociate in these compounds   The heat of ionization of a group is not only a function of the group and of the temperature, but also of the paH at which it dissociates   In the dihalogenated substitution products of l tyrosine, the hydrogen ion activities, at which the ammonium ion and the

hydroxyphenyl group dissociate, are so different than in other compounds which have been studied, that it is apparently impossible to draw conclusions from the apparent heats of ionization, calculated from the values of K_2' and K_3' at different temperatures

SUMMARY

1 The solubilities and differential heats of solution of d-tyrosine, dl-tyrosine, diiodo-dl-tyrosine, dibromo-l-tyrosine (hydrated), dibromo-l-tyrosine (anhydrous), and dichloro-l-tyrosine (hydrated) have been determined

2 Evidence has been advanced that dl-tyrosine is a compound

3 From the solubility determinations at various acidities, the apparent acid and basic dissociation constants of dibromo-l-tyrosine and dichloro-l-tyrosine have been determined at 25° and 40°C   From these data the apparent heats of ionization have been calculated

4 The question concerning which of the groups in l-tyrosine and its dihalogenated substitution products is responsible for each dissociation constant has been discussed

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VITAMIN A IN EYE TISSUES

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Animals deprived of vitamin A become night blind after exposure
to bright light, they fail to see at intensities which still readily stimu-
late the normal eye This is due to a delay in the dark adaptation
processes which normally replace the visual purple bleached by light [1]
Fridericia and Holm (1925) and Tansley (1931) have demonstrated
directly that in rats suffering from avitaminosis A visual purple is syn-
thesized more slowly than in normal animals In extreme cases none
may be formed in the retina at all (Tansley, 1933)

The dependence of visual purple formation upon vitamin A is most
direct About 3 weeks of vitamin A deprivation may result in severe
night blindness in man (Aykroyd, 1930) and in the rat (Holm, 1925)
before any other deficiency symptom can be recognized In both
animals a small quantity of cod liver oil may cure the disorder within a
day

[1] Blegvad's paper upon the relation between vitamin A and various eye ab
normalities (1924) is classic in this field. A more detailed treatment of night blind
ness may be found in the pioneer work of Kubli (1887) and a recent review by
Dieter (1931)

The intimacy of this relation suggests that vitamin A is involved directly in visual purple synthesis, and so should occur in the eye tissues   Several investigators have attempted to identify it there

Holm (1929) has shown that fresh calf retinas added to an otherwise vitamin A-free diet may cure rats suffering from avitaminosis A   Yudkin, Kriss, and Smith (1931) have obtained similar results with dried pig retinas   Such curative activity is not limited to vitamin A, however, but is shared to a high degree by kryptoxanthin (Kuhn and Grundmann, 1933) and the three isomeric forms of carotene (Kuhn and Brockmann, 1933) among the natural carotenoids   Yudkin, Kriss, and Smith also found that ether extracts of the pig retina yield a blue color with arsenic trichloride   This reaction again is not specific, but is given by the carotenoid pigments generally   The presence of vitamin A in the eye tissues is therefore still to be demonstrated

The present experiments show that considerable quantities of vitamin A occur in the retinas and the combined pigment epithelia and choroid layers of frogs, sheep, pigs, and cattle

Experiments

Eye tissues were washed twice in saline, and then repeatedly in distilled water until no hemoglobin remained in the washings   About 15 gm fresh weight of tissue were mixed with 10 cc of 95 per cent alcohol and 1 cc of 10 N aqueous potassium hydroxide, and kept at 75°C for 20 minutes to saponify   The tissues were completely disintegrated by this process   The mixture was cooled, diluted with water, and refluxed with benzine at 60°C for 20 minutes   The benzine layer was drained off, and the aqueous layer extracted cold a second time with benzine The combined benzine extracts were washed thoroughly with distilled water, then dried overnight with anhydrous sodium sulfate   The benzine was distilled off under reduced pressure, and the yellow, oily residue taken up in a few cubic centimeters of dry chloroform and stored under argon at 0°C   This solution was employed in the following tests

Antimony Trichloride Reaction —Extracts of various eye tissues, mixed with a saturated chloroform solution of antimony trichloride, always yielded the blue color characteristic of the carotenoids (Carr and Price, 1926, von Euler, Hellstrom, and Rydbom, 1929)   This faded almost completely within several minutes, the solution finally turning a permanent red

The initial blue color is due to selective absorption of the longer wave

lengths of the visible spectrum, specific for each carotenoid   In this test vitamin A may be distinguished easily from all the other known members of the group through its absorption band at about 620 mμ, since no other natural carotenoid yields bands above 590 mμ (von Euler, Karrer, Klussmann, and Morf, 1932)   The vitamin A absorption at 620 mμ falls rapidly after the antimony trichloride has been added, while in impure preparations secondary bands appear at shorter wave lengths, which cause a red color in later stages of the reaction (Wokes, 1928)

Retinal and pigment layer extracts from frogs, sheep, pigs, and cattle display all of these characteristics   The absorption band at about 620 mμ is always sharply defined   A very faint additional band at about 580 mμ is sometimes observed in more concentrated preparations   This may be due to some hepaxanthin, which always accompanies vitamin A in fish liver oils (van Eekelen, Emmerie, Julius, and Wolff, 1932, von Euler, Karrer, and Zubrys, 1934)   or to an oxidation product of the vitamin (Brockmann and Tecklenburg 1933)   Neither substance appears to possess vitamin activity   The permanent red color which succeeds the initial blue coincides with the appearance of a series of bands, the strongest at about 500 mμ, and others at about 530 and 560 mμ These are of no special interest, since a number of organic substances yield delayed red colorations with antimony trichloride (Levine and Richman, 1933)

The spectrogram of the antimony trichloride reaction with an extract of cattle retinas shown in Fig 1 illustrates these properties   For comparison a similar photograph of this reaction with halibut liver oil was taken upon the same plate   The two spectra are obviously identical

Absorption Spectrum —Chloroform solutions of vitamin A possess a single broad absorption band in the near ultraviolet, the maximum of which is at 328 mμ The remaining natural carotenoids—except hepaxanthin, with a band at 270 mμ—all have absorption bands in the visible spectrum, lying between 400 and 500 mμ (von Euler, Karrer, Klussmann, and Morf 1932)   The 328 mμ band thus distinguishes vitamin A clearly from the other carotenoids   Absorption spectra of the extracts in chloroform of sheep and ox retinas and pigmented layers and of pig retinas are shown in Fig 2   The extinction coeffi

cients for each preparation have been multiplied by a factor, to give all of the maxima the same height on the ordinates   This is tantamount to bringing all the preparations to an equivalent concentration, since concentration is directly proportional to the extinction coefficient

The specific vitamin A absorption at 328 mμ dominates all of these spectra   The curves rise without inflection from 500 mμ on, indicating
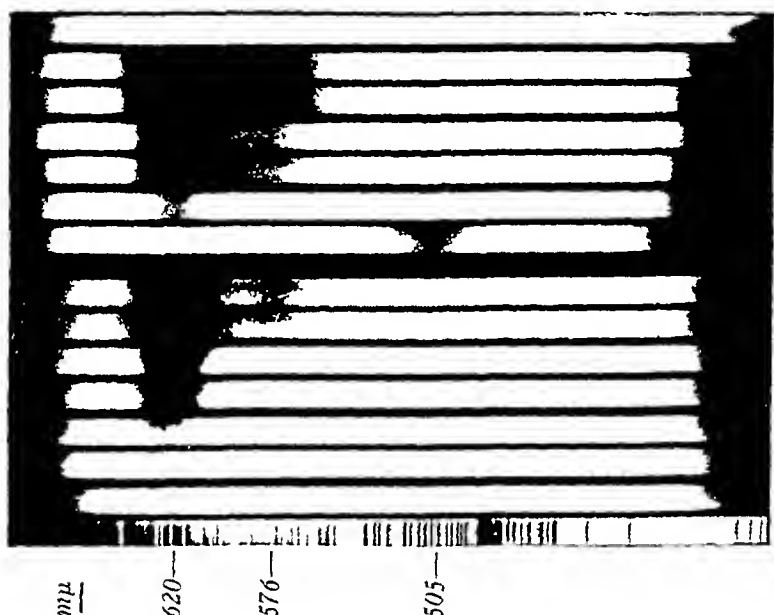
FIG 1 Spectrograms of the antimony trichloride reaction with an ox retinal oil (above) and with halibut liver oil (below)   Each series consists of 5 second exposures made at 5, 20, 32, 60, 120, and 240 seconds (reading from the top down) after mixing the reagents   Both show the 620 and 580 mμ bands fading as the reaction proceeds, and the retinal series also the growth of a secondary absorption at about 505 mμ The first and next to last spectra are of the antimony trichloride solution alone, the last is that of the iron arc

that no other known carotenoids, excepting possibly hepaxanthin, are present in the extracts from these tissues

Feeding Experiments —These were performed at the Pharmacological Institute of Hoffmann-La Roche, Basel, Switzerland, using an oil from ox retinas

2,000 ox retinas had been collected in 95 per cent alcohol over a period of 3 weeks   The solid material was centrifuged out, mixed with about 800 gm anhydrous sodium sulfate to dehydrate, and was extracted repeatedly with benzine

The alcoholic mother liquor was concentrated and similarly extracted   The combined extracts were brought into methanol, and large quantities of sterols were frozen out in solid carbon dioxide acetone mixture   The residue from the first freezing was redissolved in methanol and refrozen   The filtrates from both freezings were kept separate   They were brought into benzine, washed, and dried over sodium sulfate   After distilling away the benzine the oily residues were sealed in high vacuum   Two sterol free preparations resulted, with cod liver oil (c l o) values of 28 and 20   The total vitamin A content in all fractions measured with a Lovibond tintometer, was 2 12 mg

Rats had been kept on a vitamin A free diet until deficiency symptoms appeared loss of weight obvious reddening of the conjunctiva avoidance of light, and in some cases turbidity of the cornea (xerophthalmia)   They were then fed small quantities of the retinal oil daily   Their weights were measured in 3 day intervals

The first preparation (28 c l o units) was administered in daily doses of 1 5 mg   After 14 days the xerophthalmia had entirely disappeared, and the animals were again growing   Smaller dosages were not tried due to lack of material

The second preparation (20 c l o units) cured the avitaminosis completely in daily doses of 1 mg , 0 3 mg was found to be insufficient   Intermediate dosages were not attempted   The purest vitamin A preparations test at about 10,000 c l o units (Karrer and Morf, 1933)   It follows that this retinal oil contained about 0 2 per cent, and the minimal curative dose between 0 6γ and 2γ, of pure vitamin A

Karrer's sturgeon liver preparations maintain growth in normal animals in daily doses of 0 3γ   From 0 5 to 2γ per day of other fish

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[2] The units are those recommended in the Report of the Cod liver Oil Colour Test Sub committee, Pharmacopoeia Commission Reports, London, March 1931 (cited from Karrer, von Euler, and Schöpp, 1932)   The c l o unit is defined in terms of the blue value of the antimony trichloride coloration, measured in a Lovibond tintometer, by the formula

$$\text{C L O units} = \frac{20 \times \text{blue value}}{\text{mg oil per cc. chloroform solution}}$$

Since highly purified vitamin A preparations have a c l o value of about 10 000 (Karrer von Euler, and Schöpp, 1932, Karrer and Morf, 1933), the concentration of vitamin A in an oil may be computed in absolute units from the same formula   Substituting 10 000 for the left hand expression the denominator of the right hand one becomes milligrams of vitamin A per cubic centimeter of solution

liver oils having comparable c l o values are needed to produce the same effect (Karrer, von Euler, and Schopp, 1932) The requirement for simple maintenance appears to be less than for curing avitaminosis The retinal and the purified fish liver preparations are therefore in good quantitative agreement

Concentrations —Two non-biological methods are in standard use for measuring vitamin A concentrations The first depends upon the blue color produced with antimony trichloride and measured as already described in the Lovibond tintometer The second method uses the extinction coefficient at 328 mµ as the direct measure of concentration A 1 per cent chloroform solution of the purest vitamin A preparations

TABLE I

Quantities of Vitamin A in Eye Tissues

Animal	Tissue	Dry weight per tissue	Method	γ Vitamin A per tissue	γ Vitamin A per gm dry tissue
		mg			
Ox	Retina	51	Absorption, 328 mµ	1 05	20 6
	Retina		Lovibond	1 06	20 7
	Retina		Lovibond	1 02	20 0
Sheep	Retina	26	Absorption, 328 mµ	0 65	25 0
	Pigmented layers	32	Absorption, 328 mµ	0 75	23 5
Pig	Retina	21	Absorption, 328 mµ	0 51	24 2
	Retina		Lovibond	0 36	17 4
Frog (*R esculenta*)	Retina	3 0	Pulfrich	1 25	415
	Pigmented layers	2 25	Pulfrich	4 24	1890

has an extinction coefficient at 328 mµ of about 1350, when in a layer 1 cm deep (Heilbron, Heslop, Morton, and Webster, 1932) Since the extinction coefficient is directly proportional to both the concentration and the depth of the absorbing layer, the concentration of any unknown vitamin A solution may be computed from these figures

Measurements of both these types are presented in Table I At the time the frog extracts were prepared no Lovibond tintometer was available, in this case the absorption of the 620 mµ band was measured in a Pulfrich photometer (Zeiss), and then reduced to Lovibond units (van Eekelen, Emmerie, Julius, and Wolff, 1932)

Two features of the data are of interest The first is the constancy

of the proportion by weight of vitamin A in the mammalian tissues
The quantity of vitamin A per gram dry weight of retina in the various
species is in all cases about 22γ
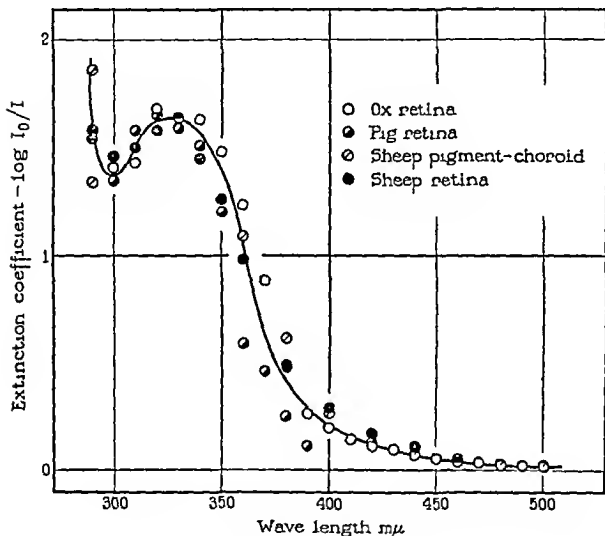
FIG 2 Absorption spectra of eye tissue extracts in chloroform The absorption is plotted as the extinction coefficient $\log I / I$, in which I is the intensity of monochromatic light entering the test solution, I that leaving it   All of the curves possess the vitamin A maximum at 328 mμ The rise at shorter wave lengths is due to an unknown component   These spectra resemble throughout those of cod liver oils (Drummond and Morton 1929)

The frog retina weighs about 1/17 as much as an ox retina, yet
contains considerably more vitamin A   The frog pigmented layers
exceed even this high concentration by about 4 5 times, and are among
the very richest animal sources of the vitamin

It is of some interest to compare these figures with the previous dat

obtained by feeding rats with whole retinas   Holm (1929) found that 50 mg of fresh calf retina displayed definite anti-xerophthalmic effects, while 750 mg also restored optimal growth   Holm states the dry weight of these tissues to be about 12 per cent of the fresh weight   Computation of these quantities on the basis of 22γ vitamin A per gram of dry tissue yields the values $0\ 13\gamma$ vitamin A for a positive effect and $1\ 98\gamma$ for complete cure, which are consistent with the results of the present experiments

Yudkin, Kriss, and Smith (1931) found 30 to 50 mg per day of dried pig retina sufficient to cure rats suffering from avitaminosis A, 20 mg per day maintained growth in normal animals, though inadequate for cures   Computed in the same way these amounts reduce to daily doses of $0\ 66$ to $1\ 1\gamma$ vitamin A for curing diseased rats and $0\ 44\gamma$ for maintaining normal animals, figures which agree well with those obtained in the present work and occurring elsewhere in the literature

This correspondence of the results obtained with whole retinas and with concentrates is an assurance that the vitamin A content of the retina accounts completely for its anti-xerophthalmic and growth-stimulating activity

Retinal vitamin A concentrations might be expected to vary with the state of nutrition   I can say little concerning this matter as yet   Animals obviously suffering from lack of the vitamin have not been used in any of these experiments   The three cattle measurements were made in January, February, and June of 1933, the change from winter to summer feeding seems to have had no effect upon the retinal vitamin A

An experiment with frog tissues is perhaps pertinent to this problem   The frogs (*R esculenta*) were winter animals   They had been brought to the laboratory in November, and kept there at room temperature without food for about 3 months   Extracts were prepared of the combined retinas and pigmented layers and the livers of five of these animals   The liver extract was bright yellow, it gave a strong antimony trichloride reaction, showing the vitamin A band at 620 mμ and a very faint one at about 590 mμ

The extracts from both organs were diluted so that the initial colorations with antimony trichloride were of equal intensity   From the dry weight of tissue used in each case and the final volumes of the solutions the relative vitamin A could be computed   The proportion

by dry weight of vitamin A in the eye tissues was found to be slightly more than 35 times that in the liver

DISCUSSION

The specific activities of vitamins are still so mysterious that their functions are at present usually referred vaguely to the whole organism The presence of vitamin A in high concentrations at the site of its most sensitive deficiency symptom—night blindness—implies some more detailed relationship This association does not seem to be merely fortuitous, for in an exhaustive survey of the tissues of the rat, Moore (1931) has shown this vitamin to be virtually absent from all other organs but the liver

Since the present experiments were first reported (1933), von Euler and Adler, at Stockholm (1934), have confirmed the presence of vitamin A in cattle retinas, but state that these and pigment layers also contain a yellow material which they believe to be carotene

I have found no trace of carotene in any mammalian eye tissue, including retinas and pigmented layers from German, Swiss, and American cattle, examined during various seasons Some yellow substance is present in the extracts from these tissues, but in no case have they exhibited spectroscopically the bands at 466 and 497 mμ characteristic of carotene, nor the band at 590 mμ which this substance yields with antimony trichloride (von Euler, Karrer, Klussmann, and Morf, 1932) The spectra reproduced in the present paper objectively confirm this observation

The concentrations of the yellow pigment, if estimated as carotene, were found by von Euler and Adler to be about 5γ per retina and 12γ per pigment layer Carotene has been shown to be about as potent biologically as an equal weight of vitamin A (Moore, 1933) Were these observations correct, therefore, cattle pigment layers should exhibit about twice the vitamin A activity of the retinas, and the latter tissues about six times the vitamin A potency actually realized

SUMMARY

1 Vitamin A has been found in the retinas and the combined pigment epithelia and choroid layers of frogs, pigs, sheep, and cattle The vitamin was identified by (a) its specific absorption at 328 mμ,

(b) the blue color yielded with antimony trichloride, associated with an absorption band at about 620 mμ, (c) anti-xerophthalmic and growth-promoting activity, and (d) quantitative relationships among the results of these three types of observation

2 The mammalian retinas contain about 22γ, the frog retinas about 400γ, and the frog pigmented layers almost 2 mg of vitamin A per gram of dry tissue

3 With the possible exception of hepaxanthin, no other carotenoids were found in the mammalian tissues

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THE RESPONSE OF SINGLE VISUAL SENSE CELLS TO LIGHTS OF DIFFERENT WAVE LENGTHS

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Any adequate theoretical explanation of color vision and its related effects involves a consideration of how the single visual sense cell responds to different wave lengths of light   Up to the present time this has been a matter of inference and hypothesis, and no direct information has been available concerning the response of the single sense cell to light from different parts of the spectrum   In an earlier paper (Hartline and Graham, 1932) we have described a method for observing nerve impulses in single fibers of the optic nerve of *Limulus polyphemus* in response to stimulation of the attached sense cells by light   The present paper is concerned with a discussion of the effect of wave length of the stimulating light upon this response

Method and Apparatus

The method for obtaining records of action potentials in the single optic nerve fibers is as follows   The lateral eye of an adult *Limulus* is excised with a centimeter or so of optic nerve and mounted in a moist chamber   With the aid of glass needles the nerve is frayed out into small bundles and the amplified action potentials in such bundles are recorded by means of an oscillograph   In several trials, splitting the bundles into still finer strands if necessary, one can obtain the response typical of a single active fiber   and locate in the eye the ommatidium which contains the corresponding receptor unit   Records from such a preparation provide the data for this report   Details of the method   the arrangement for the stimulating light [1] and the devices for controlling its constancy and the duration of its

exposure have been described in a recent paper by one of us (Hartline, 1934) In the present experiments a condition of complete dark adaptation is maintained and the temperature is controlled to within ±0 2°C

To obtain spectral lights of different wave lengths and known energy content we have employed Wratten monochromatic filters (Nos 70 to 76) in conjunction with Wratten neutral tint filters and a liquid filter of 1 per cent CuCl₂ (31 mm in thickness) to remove the near infrared (*cf* Hecht, 1928) While the Wratten filters do not yield strictly monochromatic light their transmission bands are narrow enough for the present purpose, and they have been used by other workers for a similar purpose (Hecht, 1928, Grundfest, 1932 *b*, Crozier, 1924) The transmission spectrum of each filter was corrected for the transmission of the CuCl₂ solution and the central wave length of this band was determined for each filter by the method described by Hecht (1928) [2] A direct calibration of the relative

TABLE I

Relative energies of spectral lights supplied by seven Wratten monochromatic filters The light source is a tungsten filament A filter consisting of 31 mm of a 1 per cent aqueous solution of CuCl₂ is used to remove the near infrared

Filter No	Central wave length	Relative energy
	$m\mu$	
70	690	1 72
71*A*	640	1 36
72	610	0 96
73	575	1 01
74	530	1 00
75	490	0 74
76	440	0 67

energy of the light provided by each filter was obtained by means of a thermopile and a galvanometer, the thermopile being in the position of the eye Table I gives the results of these calibrations In it are entered the central wave length and the relative energy of the light supplied to the eye when the various filters are used The energy with filter 74 has been arbitrarily assigned a value of unity

Wratten neutral tint filters were employed to vary the intensity of the stimulating lights Photometric determinations of these filters have been made several times during the course of the work and their values found to be constant within 3 per cent Moreover, they have been checked directly by means of the thermopile Under these conditions their densities with each of the monochromatic

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[2] We have neglected the correction due to the emission spectrum of the tungsten filament of the light source This will slightly affect the value of the central wave length

filters were found to be the same and within 5 per cent of the rating given by the manufacturers   Both the monochromatic filters and the neutral tint filters transmit a large amount of the near infrared   This does not affect the *Limulus* eye but of course it is measured by the thermopile and galvanometer   It is for this reason that the CuCl filter has been used in all the experiments reported in this study

RESULTS

The response of the single receptor cell in the eye of *Limulus*, in terms of impulses discharged in its attached nerve fiber, has been described in two previous publications (Hartline and Graham, 1932, Hartline 1934)   When stimulating lights of different wave lengths but of approximately equal energy content are used it is found that the response to green light is stronger than the response to either red or violet, *i e*   the latent period is shorter, the initial and maximum frequency is higher and, for short flashes, the total number of impulses is larger, with prolonged exposure the final level of frequency is higher   These are all characteristic of higher intensity of stimulation, and hence it should be possible to make up for the lower level of response in the red and violet by supplying more energy at these wave lengths   This has been done as shown in Fig 1   In this figure the intensities for the different wave lengths have been so adjusted that the responses are approximately equal   The first column gives the central wave length of the stimulating light, the second column gives the relative energy content of the light (referred to filter 74 as unity), and the right hand column contains records of the responses to a short flash (0 04 second) of each of these lights   The response consists of a burst of seven impulses (plus or minus one) and it is seen that the latent periods and frequencies are approximately the same   It is clear that when the intensities are properly adjusted there is no effect of wave length *per c*

To test this point more carefully we have chosen three spectral lights in the red, green and violet portions of the spectrum and we have taken pains to adjust their intensities to yield responses as nearly identical as possible   The close adjustment of intensity was obtained by varying the current through the tungsten filament of the light source and the energy values were obtained by direct calibration with
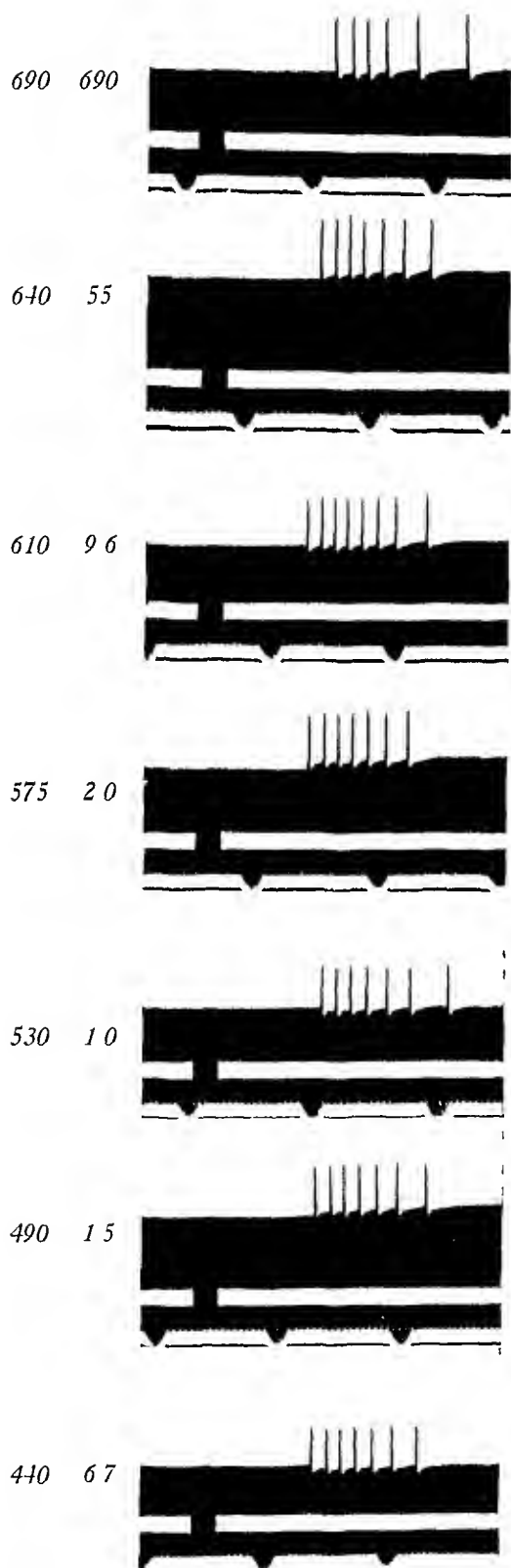
$\lambda_{m\mu}$	I	Response

FIG 1

the thermopile The responses for the three different colors agree with each other impulse for impulse as closely as the reproducibility of the results will allow The experimental findings are summarized in Table II In this table the duration of the exposure, the central wave length, and the relative energy are given in the three left hand columns In the upper part of this table are given results obtained with a flash of 0 04 second duration The features of re sponse measured are latent period, initial frequency (impulses 1 to 3), maximum frequency, and total number of impulses in the initial burst The lower part of the table summarizes results obtained with prolonged illumination In it are entered measurements of latent period, initial frequency, maximum frequency, the level of frequency reached after 3 seconds, and the time of the fiftieth impulse The experiments with the short flash were performed at two different levels of intensity, one level having one hundred times the energy value of the other

It may be seen that the responses to the three wave lengths whose energies have been properly adjusted are in close agreement, and that this agreement is maintained regardless of the energy level or duration of the illumination The agreement between the responses to the three wave lengths is as close as could be obtained at any one wave length with a repetition of the same stimulus To show the marked effect of intensity upon these features of the response we have added in Table II a a summary of results obtained in the same experiment with one wave length (filter 74) at different intensities Reference to Table II a shows within what small limits of intensity we have suc ceeded in matching the responses in Table II

Table II shows that the responses to different wave lengths may be equated at two different levels of the response and that the relative energies of the different wave lengths are in the same ratios regardless of the level of the response Thus, in Table II the energies of the

TABLE II

Comparison of matched responses to lights of different wave lengths, together with the relative energies necessary to produce them   The comparison with short flashes (0 04 sec) at two levels of intensity (2 0 log units apart) and with prolonged illumination   The energy of the green light (filter no 74) of highest intensity is assigned a value of unity

Exposure	Central wave length	Logarithm relative energy $\mathrm{Log_{10}}\,I$	Latent period	Initial frequency	Maximum frequency	Number of impulses (first burst)	Frequency at 3 sec	Time of fiftieth impulse
sec.	mμ		sec	per sec	per sec		per sec	sec
	640	$\bar{1}$ 64	0 329	39	39	7		
	530	$\bar{2}$ 00	0 325	39	39	7		
	440	$\bar{2}$ 64	0 356	38	38	7		
0 04								
	640	1 64	0 121	78	98	29		
	530	0 00	0 112	78	97	30		
	440	0 64	0 123	79	96	29		
	640	$\bar{1}$ 64	0 246	44	65		14 7	2 08
∞	530	$\bar{2}$ 00	0 238	47	64		14 7	2 42
	440	$\bar{2}$ 64	0 251	43	63		14 6	2 52

TABLE IIa

Data from the same experiment as Table II, showing the effect of intensity of light of a given wave length (filter no 74) on the various features of the response

Exposure	Central wave length	Logarithm relative energy $\mathrm{Log_{10}}\,I$	Latent period	Initial frequency	Maximum frequency	Number of impulses (first burst)	Frequency at 3 sec	Time of fiftieth impulse
sec	mμ		sec	per sec	per sec		per sec	sec
		$\bar{3}$ 60	0 409	27	27	6		
		$\bar{2}$ 00	0 323	42	42	7		
		$\bar{2}$ 30	0 267	56	56	9		
0 04	530							
		$\bar{1}$ 60	0 134	73	88	24		
		0 00	0 109	78	100	31		
		0 30	0 094	87	106	38		
		$\bar{3}$ 60	0 262	35	58		11 4	3 40
∞	530	$\bar{2}$ 00	0 238	47	64		14 7	2 42
		$\bar{2}$ 30	0 198	57	74		17 3	1 46

brighter flashes are all 100 times (2 log units) greater than the energies of the less bright flashes at the corresponding wave lengths, but it is to be noted that the responses which are matched at one level are still matched at the other level Obviously, no Purkinje effect is exhibited by the single visual sense cell of *Limulus* This is in agreement with all our findings in thirteen other experiments over even greater ranges of intensity, the curves relating magnitude of response to log intensity of stimulus are parallel for all wave lengths These experiments show that within the limits of the reproducibility of results there is no specific effect of wave length other than one of brightness

The relative energies of lights of different wave lengths required to produce the same response yield the visibility curve for the single visual sense cell In accordance with established usage the reciprocal of the energy at a given wave length necessary to produce a constant response is defined as the visibility at that wave length (Hecht and Williams, 1922) Thus in Fig 1 the reciprocals of the values of intensity in the second column are the visibility values of the light whose central wave length is given in the first column (approximate only, since the responses are not perfectly matched)

We have obtained visibility curves for sixteen single sense cells in six animals In Fig 2 is plotted the logarithm of the visibility against the different wave lengths from these different experiments The three sets of points are the values for each of three single sense cells, the curve is drawn through the average values of all sixteen experiments The detailed method for obtaining visibility values of the different wave lengths was as follows For each spectral light the combination of Wratten neutral filters was selected which would give an approximate equality of response These lights were presented in random order and records of the responses obtained Short flashes were used (0 04 second) The green filter (No 74) was chosen as a control stimulus and repeated more frequently than any of the others The effect of intensity at a given wave length, *i c* green, was also obtained over a range which would embrace the range of response inequality (Ordinarily an intensity series covering a range of 1 log unit is sufficient for this purpose) Some convenient feature of the response was chosen (*c g*, latent period, frequency of first 5 impulses, *etc*) and this feature was measured for all the responses That portion of the experiment

showing least variation in the control response was chosen and the responses to each wave length were averaged   These values are only approximately matched   To obtain the visibility it is necessary to
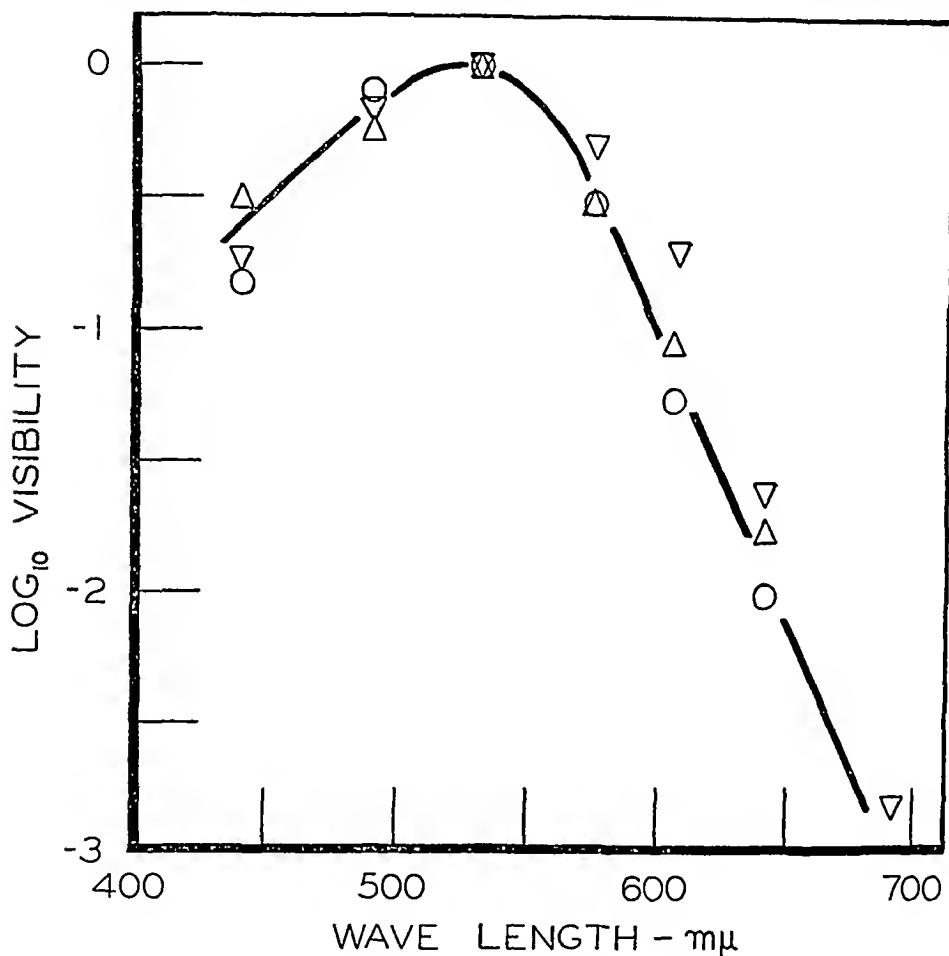
FIG 2 The logarithms of the visibility for single visual sense cells plotted against wave length   The curve is drawn through the average values from sixteen experiments   The points are values of the visibilities for single sense cells The circles and base-down triangles are the values for two cells from the same eye The base-up triangles are values for a sense cell from another eye   The visibility of green light (λ530 mμ) is assigned a value of unity

know what energy of stimulus should have been used to give a response which would be exactly matched for all wave lengths   From the curve relating magnitude of response to intensity we determined the amount

of energy by which the actual stimulus should have been increased or decreased to equal exactly the average control response   This is valid because the curves relating response and logarithm of energy for all wave lengths are parallel as has been shown   We thus obtain the exact energy at each wave length necessary to produce a given constant response   The value for the green is arbitrarily assigned a value of unity, and the reciprocals of the relative energies in the other wave lengths yield the visibilities at these wave lengths in terms of a visibility of unity for green   In the light of the previous discussion it is clear that it makes no difference to the final result what feature of the response or what level of intensity is chosen for the calculation   In deed, in some experiments a whole intensity curve was obtained for each wave length   As has been stated above, these curves, on a logarithmic scale, are parallel for all wave lengths, and the amount that each is displaced from the green gives the logarithm of the visibility (Hecht, 1928, Chaffee and Hampson, 1924)

In Fig 2 it is seen that the visibility curves for different experiments do not agree at their extremes   This is not surprising when different animals are used, but in Fig 2 two of the sets of points are from different animals in the same animal, and their lack of agreement is greater than the limits of error   This indicates that light of a given wave length does not have exactly the same visibility in all of the sense cells in the eye of *Limulus*   We have performed nine experiments which agree in indicating a true differential sensitivity for wave length among receptor cells in the same eye

There are two methods for testing this differential sensitivity   Instead of dissecting the nerve bundle until it contains a single active fiber we choose a strand in which there are several active fibers   In the first method an analysis of the relative effects of wave lengths in different receptor cells depends upon the fact that the impulses in the different nerve fibers can be identified by their characteristics of form and magnitude   The whole region supplied by the active fibers is illuminated by lights of different wave lengths whose energies have been adjusted to give approximately matched responses   In effect this amounts to performing several experiments simultaneously upon sense cells located close together under conditions which are pre sumably identical   Records from such an experiment are reproduced in Fig 3
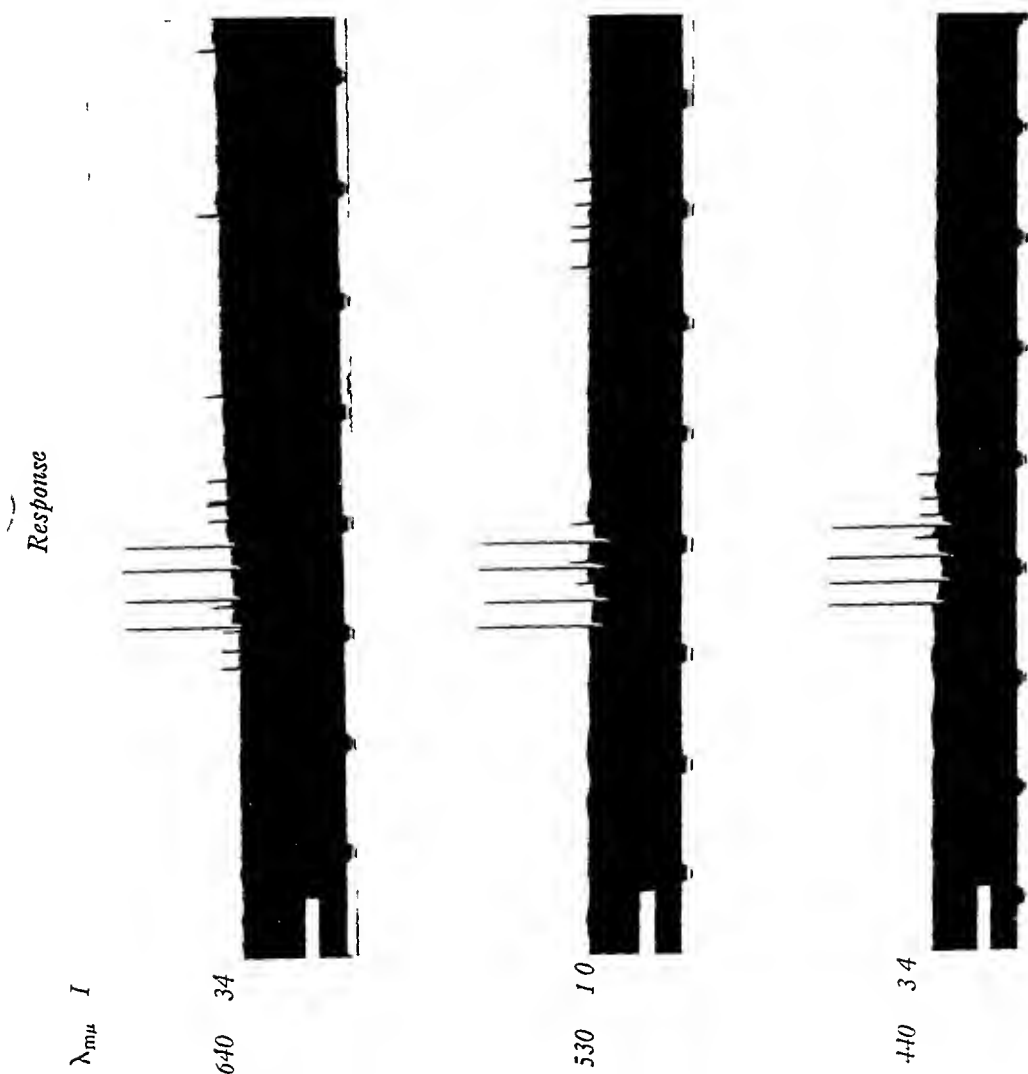
Fig 3 Oscillographic records of responses from two sense cells in the same eye to three different wave lengths   The relative intensities have been adjusted so that the responses of one sense cell (large impulses) are approximately matched   Recording as in Fig 1

In this experiment there are two active fibers whose impulses are readily identifiable by their size and form   The intensities have been so adjusted that the response in the fiber giving the large impulses is constant for all wave lengths   It is seen that these intensities do not constitute a match for the fiber giving the small impulses, but that this fiber gives a stronger response with the red light   In passing to the green and violet it is seen that the latent period progressively increases and the number of impulses decreases   The visibility for this sense cell, then, must be lower in the violet and higher in the red than the visibility for the cell giving the larger impulses

With this method high intensity responses are unobtainable since the illumination must be spread over a considerable area   Threshold

TABLE III

Negative logarithms of relative visibilities in the red and violet (basis of visibility of λ 530 mμ equal to unity) for four different sense cells in the same eye

Sense cell No	λ 640 mμ	λ 440 mμ
I	$\begin{cases} 1\ 70 \\ 1\ 75 \end{cases}$	$\begin{cases} 0\ 55 \\ 0\ 48 \end{cases}$
II	1 67	0 43
III	1 89	0 37
IV	1 98	0 81

* The two values for this sense cell are repetitions at the beginning and end of the experiment

responses are apt to be quite variable and the impulse discharge (as shown in Fig 3) is irregular   Moreover, the records are analyzable only provided they contain few active fibers   For these reasons we have employed a second method   This method depends upon the fact that the active fibers in a small bundle from the optic nerve frequently come from sense cells located in different ommatidia   It is possible to locate these ommatidia and illuminate them separately with a small intense spot of light (Hartline and Graham, 1932)   By means of an improved micrometer manipulator (constructed by Mr A J Rawson of this Foundation) similar to the one described in an earlier paper (Hartline and Graham, 1932) it has been possible for us to examine as many as six separate sense cells in the same eye in rapid

in the same eye are approximately similar they do differ by significant amounts   It is true that many of the visibility values fall close to the average, but a good number deviate appreciably   In view of the evidence we do not believe that this can be ascribed to experimental error and feel that it represents a true differential sensitivity to wave length among the sense cells of the eye   In view of the primitive nature of the *Limulus* eye this finding is somewhat surprising, for it is, of course, precisely such a mechanism as is postulated to explain color vision in the higher animals (*cf* Hecht, 1930)   While any single sense cell cannot distinguish wave length differences it is clear that, for example, the two sense cells whose responses are given in Fig 2 can together distinguish violet from red, and the presence of differential sensitivity to wave length in the *Limulus* eye may be considered a possible peripheral mechanism for color discrimination   Whether the animal possesses the adequate central and motor equipment to make use of this mechanism is not known

The present data do not allow us to discuss in detail the types of variation in the visibility curves   Apparently, however, the variations are not confined to any particular portion of the range of wave lengths but are to be observed over the entire curve   Moreover, the different curves cannot be obtained from a single curve by a shift in the position of the maximum   We have been unable to distinguish any tendency on the part of the curves to fall into groups within which the visibility curves are identical or even nearly so   As to the causes which might underlie the differences in the visibility curves of the various sense cells whether, for example, they are due to overlying pigment or to slight differences in the photosensitive substance itself, we are not in a position to speculate

SUMMARY

The effect of various wave lengths of visible light in the stimulation of single visual sense cells has been studied by means of the single fiber preparation from the eye of *Limulus*   Oscillographic records were made of the impulse discharge in a single optic nerve fiber in response to stimulation of the attached sense cell by lights of different wave lengths   Wratten monochromatic filters supplied the means for

obtaining the different spectral lights, the total intensity supplied to the eye being determined by a thermopile and galvanometer

With lights of approximately equal energy content the strongest response occurs to the green region of the spectrum   The response, however, does not vary qualitatively with wave length   By the proper adjustment of intensity, responses can be obtained which are identical, impulse for impulse, for all the spectral lights used   Moreover the ratios of the intensities for the various wave lengths necessary to produce a constant response do not vary with the intensity level of the stimulating lights, there is no Purkinje effect   The single visual sense cell can gauge brightness but cannot distinguish wave length

The reciprocals of the intensities necessary to produce a constant response when plotted against wave length give the visibility curve for the single sense cell   This curve is symmetrical about a maximum at λ520mμ, falling off to low values in the red and violet   It closely resembles the visibility curve for human rod vision

Bundles from the optic nerve containing several active fibers whose impulses can be distinguished by differences in form and magnitude or whose attached sense cells can be located and illuminated independently were used to determine whether there is any differential sensitivity among sense cells in the same eye for different regions of the spectrum   Such a differential sensitivity has been found to exist in the eye of *Limulus* and may be considered a peripheral mechanism of color vision

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regarded from different standpoints, not two different processes or processes in two different membranes   The question, therefore, as it seems to us, is not, which of the two processes occurs, but rather which of the two viewpoints is preferable, in other words which viewpoint offers most chance to clarify the great complexity of permeability, that becomes more and more evident as more and more data in this field are collected

For this question the following considerations will prove fruitful   A solution, in the thermodynamic sense, is a homogeneous system, $i\,e$, characterized by the fact that each small fraction of volume has the same properties as the system as a whole   If the concept of solution is to be used, this condition must therefore be fulfilled   It must be true that the inhomogeneity of matter due to its molecular structure is irrelevant   Now the work of Langmuir, Adam, Rideal (1) and others has shown that at interfaces between two phases very strong molecular forces are active, resulting in distinct orientations of the molecules Systems under such forces thus differ widely in the properties of their different parts   According to Langmuir's interpretation of the liquid expanded films (9) they may differ even in the state of aggregation The space properties of the molecules, which cancel out in a homogeneous system due to random distribution, here add up due to orientation and become the outstanding properties of the system   It should be strongly emphasized that this not only holds for monomolecular films, but for all interfaces where polar molecules are involved   In other words   At interfaces such as the cell membrane properties come up, which are due to a definite structure, and which do not obtain in the bulk of a homogeneous solution

A very striking example of such structural properties due to orientation is the ion permeability of crystals   The investigations of Tubandt and others (27) have shown that most electrolyte crystals have a distinct selective ion permeability   For instance, crystals of AgCl, AgBr, Ag_2S, NaF, and others are solely cation permeable, $PbBr_2$, BaF_2, $BaCl_2$, and others solely anion permeable   According to Joffé the limiting size of ions which can diffuse through a crystal depends on the crystallographic direction in which the diffusion takes place   It is obvious that at least for the latter observation the molecular structure is responsible and to regard the crystal as a solvent would be inadequate   The common feature which connects this field with that of cell permeability is the decisive effect of an oriented, instead of a random, molecular structure

For this reason it seems preferable not to use the concept of a homogeneous solution for the interpretation of cell permeability, but to base the interpretation on molecular theory. This can be done by regarding the intermolecular spaces as pores and deriving the conditions in these pores from the properties and force fields of the adjacent molecular groups

In this paper the attempt will be made to apply the points of view discussed to the collodion membrane as a model for cell permeability. Taking into consideration recent advances in knowledge concerning molecular structures and new experiments on the reversal of the charge of the membrane, and furthermore experiments on membranes made of other cellulose derivatives, the mechanism of potentials of this membrane will be discussed once more. It will be shown that its properties can be understood best from its molecular structure, furthermore that, using this interpretation, the contrast between solubility mechanism and pore mechanism largely disappears. Finally asymmetric membranes will be described and discussed

The Mechanism of the Membrane Potentials of the Collodion Membrane

The entirely dried collodion membrane resembles the cell membrane to a certain extent as to its permeability and its electromotive properties. It allows molecules of non electrolytes up to a similar order of size to permeate as the membrane of the erythrocyte does (15, 18) Its behavior towards electrolytes is that of a selective ion permeable membrane (11, 16), as has also been shown for different cell membranes, for instance that of the erythrocyte, the nerve, and the muscle

The mechanism of this selective ion permeability has been interpreted by Michaelis in the following way. The membrane consists of electrically non conducting matter. Its electrical conductivity between electrolyte solutions is due to the presence of pores, through which the solution penetrates. Within these pores the cations have a higher apparent relative mobility than anions. If the membrane is put between two solutions of the same electrolyte in different concentrations, a diffusion potential arises, which is positive on the side of the dilute solution [1]. The drop of potential extends throughout the

[1] We shall designate the concentration potential as positive throughout this paper if in the external circuit it is positive on the side of the more dilute solution and vice versa

length of the pore   Its amount can be calculated by application of
Nernst's formula for the diffusion potential

$$E = \frac{RT}{F} \frac{u - v}{u + v} \ln \frac{c_1}{c_2}$$

where u and v mean the apparent relative mobilities of the cations and
anions, c_1 and c_2 the activities of the electrolyte on the two sides of the
membrane   The direct experimental determination of the transfer-
ence numbers (17) in an electric field for a given membrane agreed
satisfactorily with those calculated from the measured concentration
potentials

The reason for the higher relative cation mobility was assumed to be
an adsorption and thereby immobilization of the anions at the walls
of the pores   Accordingly, the narrower the pore is, the higher is the
fraction of adsorbed anions, the higher the difference of the relative
mobilities of cations and anions, and the higher therefore the concentra-
tion potential   A pore, which is so narrow, that besides the adsorbed
anions no more anions can enter is solely cation permeable, the value
$\frac{u - v}{u + v}$ then equals 1 and the concentration potential reaches its thermo-
dynamic maximum, which is equal to the potential of a concentration
chain, reversible for cations

In contrast to this interpretation Beutner (4, 5) and Northrop (23)
have claimed that the properties of the collodion membrane are not due
to the presence and particular properties of pores, but to the respective
solubilities of permeating molecules in the substance of the membrane

Northrop, whose arguments will be discussed in detail later, comes
to the following conclusion   The ordinary collodion membrane,
which has not been entirely dried, has pores   But when the membrane
dries entirely, these pores disappear and then the membrane allows
molecules to penetrate only if they are soluble in the substance of the
membrane   According to this assumption the membrane would
change its behavior decisively, when losing the last remainder of the
organic solvent   One would expect that this more or less sudden
change would also appear in its electromotive behavior in the form of a
discontinuity in the plot of potential against permeability   This is,
however, not the case, as Fig 1 shows   The transition from mem-
branes with large pores to those with supposedly no pores is steady

The permeability of such membranes has been graded by imbibition of completely dried membranes in alcohol solutions of different concentrations according to Brown Preliminary experiments had shown that alcohol concentrations up to 85 per cent do not change the electromotive properties much The concentrations of alcohol used therefore were 85, 90, 92 93, 94, and 95 per cent. The completely dried membranes were allowed to swell in alcohol for 2 days Then their concentration potentials between N/10 and N/100 KCl and their permeability for HCl were determined The latter was done by filling them with 25 cc N/10 HCl, putting them into 25 cc of water and titrating the HCl content of the outer solu
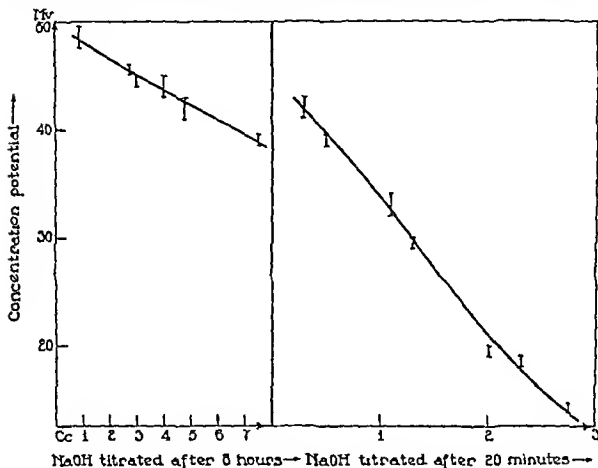
FIG 1 Concentration potentials and permeability for HCl of graded collodion membranes

tion after a certain time It was necessary to use two different times of diffusion for the less permeable membranes and for the more permeable ones, so that only within these two groups are the values directly comparable Two membranes however, are represented in both groups, so that it can be seen that no sudden change between the two groups occurs

Further evidence for the steady transition from highly permeable to completely dried membranes is the following The sign of the concentration potential of the dried collodion membrane can be

reversed, as will be shown in the next section, by impregnation with quinine   Membranes with high negative concentration potentials can thus be obtained   If now such a membrane is not allowed to dry entirely, but the concentration potential is measured after different times of drying, gradually increasing negative concentration potentials are obtained, showing that the same properties control the behavior of the wet and the dry membrane, varying only quantitatively, not qualitatively with the size of the pores   This is shown in Table I The same is true for membranes of cellulose acetate, which will be described below

We prefer the assumption, therefore, that the transition from the wet to the dry membrane does not consist of an absolute constriction

TABLE I

Concentration potentials of collodion membranes impregnated with quinine and soaked in water after different times of drying   The potentials are measured between N/10 and N/100 KCl   The sign is that of the dilute solution in the external circuit

Membrane No	Time of drying at 30°C in	Concentration potential
	min	mv
1	10	−2
2	15	−14
3	20	−22
4	35	−48

of the pores, but of a gradual transition of the pores into the intermolecular spaces, the change thus being only quantitative, not qualitative   The fact that according to Michaelis and coworkers (12, 14) besides the wet collodion membrane many other types of membranes, which doubtless have pores, such as the parchment membrane, also show qualitatively the same effect, though to a smaller extent, confirms the view that the transition from the dried membranes to the membranes containing pores is gradual   The structure of the pore system gradually changes to the crystal structure during drying

The experimental basis for this view is provided by the recent investigations on x-ray patterns of cellulose and cellulose derivatives The cellulose fibre has a crystal structure (25), in which the long cellulose molecules lie parallel   The unit cell of the crystal is the

glucose ring, whose dimensions are 5 15 Å u in the direction of the length of the fibre and 5 4 Å u and 6 1 Å u in the perpendicular directions   Trogus and Hess (26) showed that nitrocellulose fibres and fibres of other cellulose derivatives agree with cellulose in the fibre period, while the intermolecular distances are larger than in cellulose   Mathieu (10) showed that when from a nitrocellulose solution a film is formed by evaporation of the solvent, the molecules, which were entirely dispersed in the solution, rearrange themselves to a great extent, a crystal structure being formed again, whose x ray pattern is very much like that of the fibre, though usually yielding not as sharp interferences as the former

The collodion membrane accordingly has to be regarded as a crystal in the same sense as silk may be considered as a crystal   The electro static conditions in the intermolecular spaces are then determined by the properties of the adjacent molecular groups   According to the picture of the nitrocellulose molecule as shown in Fig 2, these groups are the NO_3 groups   These highly polar groups have a dipole moment, the outer end of which is negative

An adsorption of anions at these groups is not to be expected there fore   On the other hand the negative charge of the collodion mem brane may be taken as proven, as shown not only by the sign of the concentration potential, but also by the direction of electroosmosis across the membrane or cataphoresis of particles of collodion   The following modification of Michaelis' interpretation seems to be neces sary therefore

The electric field, which counteracts the entrance of anions into the pore, is not established by adsorbed anions, but by the polar NO_3 groups, that is to say by the membrane itself   The centers of negative charges in these groups lie towards the interior of the pore and thus repel anions   So within the total area of the pore there will be a certain fraction, into which, due to this repulsion, anions cannot enter, while cations can   The extent to which, due to this situation, the apparent average mobility of anions in the pore is decreased relative to the cation mobility, therefore depends on the ratio of this frac tional area to the total area of the pore   Hence the concentration potential depends on the size of the pore just as it did assuming the mechanism of an anion adsorption

A phenomenon which could not satisfactorily be accounted for by the assumption of an adsorption of the penetrating ions is the behavior of protein membranes in buffer solutions as described by Fujita (13) These membranes between two buffer solutions of the same pH, but of different concentrations, showed a concentration potential, the sign of which depended on whether the pH of the buffer was above or below the isoelectric point of the protein   In the first case the membrane appeared to be negatively charged and cation permeable, in the latter case positively charged and anion permeable   The diffusing ions were
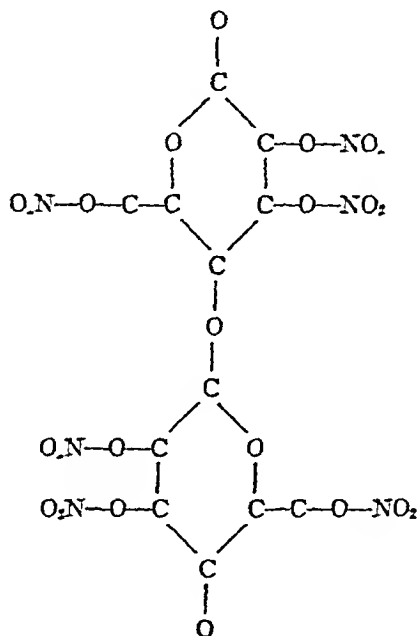
FIG 2 The nitrocellulose chain

Na$^-$ and acetate$^-$, the concentrations of the H$^+$ and the OH$^-$ ions were negligible   Hence the assumption of an adsorption of the diffusing ions controlling the concentration potential leads in this case to the assumption of an adsorption of Na$^+$ by protein   Such an adsorption compound is not known   If, however, the charge of the membrane is the charge of the protein molecule itself, depending on the pH as the latter does, and the inhibition of cation mobility is due to the repulsion of cations by this charge and not to immobilization by adsorption, the interpretation is compatible with all we know of the electric properties of proteins

Let us now consider Northrop's and Beutner's arguments against the assumption of the pore mechanism Northrop found that the rate of permeation of gases through the dry collodion membrane is not inversely proportional to the square root of the density of the gas as it holds for diffusion of gases through pores Furthermore, that the rate of gas permeation is independent of whether the membrane is filled with water or with air, whereas gas diffusion in air is about 10,000 times faster than in water Finally, that the temperature coefficient of permeation through a collodion membrane is higher than that of a mere diffusion

All this is comprehensible, if the dimensions in question are taken into account The dimensions of the cell of the cellulose molecule are around 5–6 Å u Nitration increases the intermolecular distances The order of magnitude of the pore diameter has therefore to be assumed to be several Å u Northrop himself calculated the diameter of the pore first from the rate of flow of water through the membrane to be about 10^{-9} cm , second from the vapor tension of water in the pores to be 10^{-4}–10^{-8} cm These dimensions are far below the mean free path of the molecules, therefore the number of collisions of the molecules with the wall of the pore is of higher order of magnitude than that of collisions with other molecules Hence the quoted law of gas diffusion does not hold nor is it to be assumed that the gas diffusion is inhibited by water in a similar way as in macroscopic dimensions Also the temperature coefficient of gas diffusion under these circumstances is certainly not that of free gas diffusion for the same reason It may be permissible to speak of a "solution" of the penetrating substance under such conditions, but this is by no means a contrast to any pore theory This statement holds for all cases, where the penetrating substance is not able to disrupt the crystalline structure of collodion, that is to say causes no swelling or solution of the collodion, but where even under the influence of the penetrating substance the structure remains rigid The difference between a substance like sodium chloride or sugar and on the other hand a substance like acetone is (cf 20) that the former has to utilize for penetration the preformed holes of the rigid structure, whereas the latter, when present in sufficient concentration, can disintegrate the structure by combination with the molecules of the membrane In all cases where the structure remains rigid, the concept of solubility is not very adequate and certainly not

in contrast to the pore mechanism   As long as the membrane remains rigid, the pore theory holds good whether the pores are large or of molecular size

Beutner interprets the potentials of the collodion membrane as phase boundary potentials, due to the different solubility of anions and cations in the substance of the membrane   It will be shown later that the contrast between this interpretation and that of Michaelis disappears to a great extent when they are compared in detail   At this place we shall only briefly discuss the experiments on which he bases his arguments in his last papers (4, 5)

He shook cubes of collodion with solutions of chlorides and showed that the solutions become acid   He ascribes this to the formation of HCl and compares it to the formation of free acid, which he justly assumes to account for the concentration effect in his "oil chains"   We repeated his experiments and could confirm the acidulation   It is, however, mainly due to hydrolysis of the collodion, as can easily be shown by means of the diphenylamine test for nitric acid, which is positive in the solution when it has become acid

The second experiment of Beutner's arguments was the following   He obtained a concentration effect in the chain

| / ether-alcohol solution | / solid collodion mem- | / aqueous solution of |
| / of collodion | / brane | / KCl |

by varying the concentration of the KCl solution   In this arrangement he considered the conditions for the formation of a diffusion potential not to be established and concluded that the effect could therefore only be interpreted in terms of phase boundary potentials   But, if the chain conducted the electric current, there must be in the collodion solution some electrolyte present   A diffusion potential hence must arise between the KCl solution and this electrolyte, to which we may apply Henderson's equation (8)

$$\pi = 0,0001983 \times T \times \frac{(U_{\mathrm{I}} - V_{\mathrm{I}}) - (U_{\mathrm{II}} - V_{\mathrm{II}})}{(U'_{\mathrm{I}} + V'_{\mathrm{I}}) - (U'_{\mathrm{II}} + V'_{\mathrm{II}})} \log \frac{U'_{\mathrm{I}} + V'_{\mathrm{I}}}{U'_{\mathrm{II}} + V'_{\mathrm{II}}}$$

where the subscript I refers to the electrolyte in the KCl solution, the subscript II to the electrolyte in the alcohol-ether solution   According to the low concentration of the latter the terms with the subscript II become negligible as additive values, and the equation assumes the form

$$\pi = 0,0001983 \times T \times \frac{u - v}{u + v} \log \frac{c}{U'_{\mathrm{II}} + V'_{\mathrm{II}}}$$

where c is the concentration of the KCl solution   This shows that the diffusion potential varies with the logarithm of the concentration of KCl, which means that Beutner's experimental results do not disprove the pore mechanism

If we finally compare his interpretation of the membrane potentials as phase boundary potentials with the interpretation developed here, we shall see that the contrast disappears to a great extent. The distinction between the entrance of a molecule into the intermolecular space and its dissolving in the phase of the membrane is purely formal. A difference between the pore mechanism with anion adsorption and Beutner's mechanism had been that in the latter an excess of cations over anions of the electrolyte in the membrane had to be assumed, which was not true for the pore interpretation, where anions and cations were present in equal amounts, the anions only being immobilized by adsorption. In the modified pore interpretation this difference disappears, a solely cation permeable pore according to this interpretation contains only cations.

Following this aspect of the modified pore theory into its consequences, we find more analogies to Beutner's interpretation. The question, namely, how cations can enter such a solely cation permeable pore without disturbing electroneutrality, leads to the assumption that they exchange against H ions, which means an acidulation of the external solution. Such an acidulation is also a consequence of Beutner's interpretation of the concentration effect at "oil chains."

This leads to a further point, which has constituted a difference between the two mechanisms, $i\ e$, the course of the drop of potential. In the case of a phase boundary potential it takes place mainly in two jumps at the phase boundaries, in the case of an ordinary diffusion potential it extends over the length of the diffusion gradient. Now, the more a collodion membrane approaches exclusive cation permeability, and the more therefore the ion exchange just discussed takes place, the more the course of the potential drop must approach that of a phase boundary potential. For this exchange does not lead to equal concentrations inside and outside the pore, but to an equilibrium with a low cation concentration inside. Thus besides the diffusion potential throughout the length of the pore we have two potentials of opposite sign at the ends of the pores, whose difference, together with the potential within the pore, makes up the total measured potential difference.

It can be calculated that these two latter potential differences are high as compared with the former. Let a solution of a uniunivalent strong electrolyte KA

dipole moments is correct, the behavior of such membranes should be determined by the dipole moments of the respective groups corresponding to the NO_3 group in the collodion membrane, $i\ e$, of OH in the case of pure cellulose and of the substituents in the case of cellulose derivatives

The dipole moments of various groups have been studied as to their magnitude and their direction by Williams (28)   He determined the moments of disubstituted benzene derivatives and concluded the direction of the moments of different groups from the moments of para-di-substitutes   If this was lower than that of the monosubstitute with one of the two substituents, the directions of their moments were the same, if it was higher, they were opposite   Thus he obtained the following scale for dipole moments of different groups, in which the direction of the moment is indicated by the sign

Substituent		Substituent		Substituent	
Cl	−1 5	OH	−1 7	OCH₃	−1 0
Br	−1 5	COOCH₃	−1 8	COOH	−0 9
I	−1 3	NO₂	−3 8	CH₃	+0 4
F	−1 4	COH	−2 8	NH₂	+1 5

It is interesting to see that negative groups are apparently much more frequent than positive ones   Possibly this is the reason why positively charged membranes are so rare (disregarding those with basic groups)

Membranes of cellophane, ethyl cellulose, and cellulose acetate were investigated, the supposedly acting groups being OH, OC_2H_5, and $OOCCH_3$ The moments of OH and OCH_3 are among those determined by Williams, they are both directed the same way as that of NO_2 (we used the ethyl group instead of the methyl group, but the difference between these two groups as to the dipole moment is usually small and does certainly not affect the direction of the moment)   Thus it was to be expected that the membranes of ethyl cellulose and cellophane have the same charge as collodion   The acetyl group is not in the above list (the group $COOCH_3$ in the list is the end-group of methyl benzoate), it is, however, a polar group as known from the dipole moment of ethyl acetate and methyl acetate   The magnitude of its moment is about the same as that of OH, its direction, however, cannot be predicted and has not been determined yet

The cellophane used was the commercial cellophane in sheets and cellophane tubing for dialysis   The membranes of ethyl cellulose were prepared from a 5 per cent solution in chloroform, those of cellulose acetate from a 5 per cent solution in acetone   (Ethyl cellulose and cellulose acetate were kindly given by the Du Pont Rayon Company, whom we wish to thank very much)   The latter were prepared on mercury in the usual manner, while for the ethyl cellulose membranes this procedure did not prove useful   The ethyl cellulose membranes made on mercury

had very high electric resistances mostly over 40 megohms, even when not completely dried but immersed in water after a short time of drying Some of them became more conducting with time yet out of twelve only three membranes could be used They gave qualitatively the same effect as the ones described below Those are membranes in the form of a bag prepared in the known way in a small beaker and soaked with water before complete drying

The cellophane membranes, as expected, yielded positive concentration potentials, as the collodion membrane does Their magnitude was usually 10 to 20 mv between N/10 and N/100 KCl and 20–30 mv between N/100 and N/1000 KCl, with membranes made of cellophane sheets Some, however, had higher potentials up to 40 mv The

TABLE II

Concentration potentials and chemical potentials of membranes of ethyl cellulose (bags) The sign is that in the outer solution (in the external circuit)

Solution		Potential			
		Membrane No			
Inside	Outside	1	2	3	4
		mv	mv	mv	mv
N/10 KCl	N/100 KCl	+33	+27	+32	+31
N/100 KCl	N/10 KCl	−38	−30	−33	−35
N/10 KCl	N/10 KCl		0	0	0
N/10 KCl	N/10 NaCl	+4	+4	+4	+4 5
N/10 KCl	N/10 LiCl	+9	+9	+9	+5
N/10 KCl	N/10 KNO₁		+2	+1	+1

dialyzing tubes are so highly permeable that their concentration potential is only 1–2 mv , but of the same sign

The ethyl cellulose membranes are also negatively charged, as expected The concentration potential is positive and chemical potentials are more affected by cations than by anions, as is shown in Table II These chemical potentials are, however, surprisingly low, considering the relatively high concentration potential The chemical potentials of the dried collodion membrane as well as those of the impregnated collodion membrane and the cellulose acetate membrane described below are much higher Chemical potentials and concentration potentials do not vary in parallel fashion This is compatible with our view of the mechanism of the potential, because according to

The results are shown in Table IV   The given concentration potentials are the highest that were measured   The basic dyestuffs always reverse the charge of the membrane, as also do alkaloids and phenylendiamine   Some of the measured concentration potentials

TABLE IV

Concentration potentials of impregnated collodion membranes, measured between N/100 and N/1000 KCl   The sign is that in the dilute solution (in the external circuit)

Impregnating substance	Concentration potential
	m^-
Methylene blue	−55
Toluidin blue	−28
Thionine	−25
Safranine	−40
Neutral red	−43
Brilliant kresyl blue	−16
Nile blue sulfate	−28
Basic fuchsin	−55
Rhodamine B	−44
Pyronine	−23
Eosin	+28
Uranin	+21
Picric acid	+45
Sudan	+10
Quinine base	−49
Quinine chloride	−50
Quinidine	−40
Strychnine	−28
Brucin	−35
Phenylendiamine	−27

are very close to the thermodynamic maximum   which means that the membranes are nearly exclusively anion permeable.

Acid dyestuffs do not reverse the charge   The membranes in Table IV were made of different brands of collodion   without control membranes of the same brand, so that the question whether these dyestuffs affect the magnitude of the potential cannot be answered from the

assume that the two valencies of the O atom form an angle, it seems possible that the C=O bond is turned around, the O thus facing the cellulose chain, the C the intermolecular space Thus the assumed direction of the moment would be established In favor of this assumption is the fact that hydroquinone-di acetate, though seemingly a symmetric molecule, has a high moment, which Williams accounts for by the valency angle at the O atom

Summary—1 In agreement with our interpretation of the po tentials and the known direction of the dipole moments of OH and OCH₃, cellophane and ethyl cellulose membranes are negatively charged

2 The cellulose acetate membrane is positively charged, being the first case of a non basic positively charged membrane The direction of the moment of the acetyl group is not known, but con cluding from other experimental data it seems likely that it agrees with our interpretation

The Reversal of the Charge of the Collodion Membrane

To get further experimental evidence for the mechanism discussed, we investigated the conditions under which the charge of the collodion membrane can be reversed Mond (21) has described an anion permeable membrane, which he obtained by adding to the alcohol ether solution of collodion the basic dyestuff rhodamine B, and then forming a membrane on mercury in the usual manner The anion permeabil ity was checked by the sign of the concentration potential, by the electromotive effectiveness of anions and ineffectiveness of cations, and by the analytical proof of an anion exchange Rhodamine has two ethylated NH₂ groups We therefore at first determined whether other organic bases had the same effect A series of basic dyestuffs and alkaloids were investigated as to their reversing effect on the charge of the membrane in the following manner

The dyestuff itself was dissolved in one of the commercial collodion solutions, in as high a concentration as could be reached by repeated stirring The colored solution was poured on mercury in the usual manner to form a membrane by evaporation of the solvent The charge of the membranes obtained was checked by the sign of the concentration potential between N/100 and N/1000 KCl

The results are shown in Table IV   The given concentration potentials are the highest that were measured   The basic dyestuffs always reverse the charge of the membrane, as also do alkaloids and phenylendiamine   Some of the measured concentration potentials

TABLE IV

Concentration potentials of impregnated collodion membranes, measured between N/100 and N/1000 KCl   The sign is that in the dilute solution (in the external circuit)

Impregnating substance	Concentration potential
	mv
Methylene blue	−55
Toluidin blue	−28
Thionine	−25
Safranine	−40
Neutral red	−43
Brilliant kresyl blue	−16
Nile blue sulfate	−28
Basic fuchsin	−55
Rhodamine B	−44
Pyronine	−23
Eosin	+28
Uranin	+24
Picric acid	+45
Sudan	+10
Quinine base	−49
Quinine chloride	−50
Quinidine	−40
Strychnine	−28
Brucin	−33
Phenylendiamine	−27

are very close to the thermodynamic maximum, which means that the membranes are nearly exclusively anion permeable

Acid dyestuffs do not reverse the charge   The membranes in Table IV were made of different brands of collodion, without control membranes of the same brand, so that the question whether these dyestuffs affect the magnitude of the potential cannot be answered from the

experiments shown in that table Experiments, however, with Echt-rot, an acid dyestuff of high lipoid and low water solubility, have shown that the concentration potential of an impregnated membrane is increased as compared with control membranes of the same brand The control membranes yielded concentration potentials of $+48.5$ mv, $+48.5$ mv, 49.5 mv, whereas the concentration potentials of the impregnated membranes were very close to the thermodynamic maximum 54 mv, 56 mv, 56 mv

All the reversing substances have in common a basic group, which means a group carrying a free positive charge Now the effect of a free charge is considerably stronger than that of a dipole If therefore the reversing effect consists in a counteracting of a free charge against a dipole, one would expect that to overbalance the effect of a certain number of NO_3 groups only a smaller number of basic groups would be necessary We determined the limiting concentration of quinine that must be introduced into a membrane just to reverse its charge and found, indeed, that at this concentration the number of NO_3 groups present is 1-2 orders of magnitude higher than that of basic groups This is shown in Table V The concentration potential changes its sign between the concentrations 1/30 and 1/90, that means one basic group compensates about 30-90 NO_3 groups

In membranes with quinine at concentrations near the limiting reversing concentration, the potentials often are inconsistent. The reason apparently is that the degree of dissociation of quinine is not defined in the unbuffered solutions of KCl The charge of these membranes depends on the pH At low pH, when the H association is favored it is positively charged, at high pH negatively as the ordinary collodion membrane This is shown in Table VI The membranes act like ampboteric membranes The ampboteric nature, however, is due to the counteracting of free charges and dipole moments and not, as with protein membranes, to the counteracting of free charges of opposite sign

The experiments described so far have been performed with "Kollodium Schering Kahlbaum DAB6" This brand of collodion yielded the membranes with the highest and the most consistent concentration potentials, while with other types of collodion lower and less consistent potentials were obtained Table VII shows the potentials of varie

types of membranes   This difference is certainly not due to different sizes of pores, for the membranes with low potentials often had a high electric resistance and *vice versa*   Different degree of nitration does

TABLE V

Concentration potentials of collodion membranes impregnated with quinine, measured between N/10 and N/100 KCl   The sign is that of the dilute solution (in the external circuit)   The concentration of quinine is indicated by the ratio basic groups NO₃ groups

Ratio basic groups NO₃ groups	1/10	1/100	1/1000	1/10,000
Concentration potential, *mv*	−25	+21	+23	+37
Ratio basic groups NO₃ groups	1	1/100	1/1000	
Concentration potential, *mv*	−27	+8	+43	
Ratio basic groups NO₃ groups	1/10	1/100	1/1000	1/10,000
Concentration potential, *mv*	−27	Inconsistent	+20	+35
Ratio basic groups NO₃ groups	1/10	1/100	1/1000	1/10,000
Concentration potential, *mv*	−30	+27	+44	+44
Ratio basic groups NO₃ groups	1/30	1/90	1/270	1/810
Concentration potential, *mv*	−20	+26	+39	+54
Ratio basic groups NO₃ groups	1/10	1/30	1/90	1/270
Concentration potential, *mv*	−17	−8 5	+1 5	+8 5

TABLE VI

Concentration potentials of collodion membranes impregnated with quinine, measured between N/100 and N/1000 HCl or NaOH   The concentration of quinine is given as fraction of weight of the dry membrane   The sign is that of the dilute solution (in the external circuit)

Concentration of quinine	1/10	1/30	1/90	1/270
Potential in HCl, *mv*	−56	−55	+26	+34
Potential in NaOH, *mv*	+45	+49	+48	+50
Concentration of quinine	1/10	1/30	1/90	1/270
Potential in HCl, *mv*	−35	−39	+16	+24
Potential in NaOH, *mv*	+30	+37	+38	+39

not seem, either, to be the cause   Now Mathieu has found that the rearrangement of the molecules in the films, especially in the highly nitrated ones, was very variable   Sometimes he obtained very sharp

interferences, sometimes very indistinct patterns   It is highly probable that the variability of concentration potentials is due to this variability of the arrangement of the molecules   It would be very interesting to investigate the x ray patterns of membranes with different concentration potentials   In favor of this assumption is the fact

TABLE VII

Concentration potentials of collodion membranes of various brands of collodion, measured between N/100 and N/1000 KCl

Brand of collodion	Concentration potential	
	Highest value	Lowest value
Kollodium Schering Kahlbaum 'DAB 6	56	50
Kollodium Schering Kahlbaum ' zur Analyse '	46	35
Kollodium Schering Kahlbaum ' zur Herstellung von Membranen	50	38
Collodion Merck USP '\	48	10
Collodion Mallinckrodt	25	16

TABLE VIII

Concentration potentials of membranes of 'Collodion Merck' (which gives low positive concentration potentials, when not impregnated), impregnated with methylene blue or quinine   The potentials are measured between N/10 and N/100 KCl   The sign is that in the dilute solution (in the external circuit)   The concentration of the impregnating substance is given as fraction of weight of cellulose nitrate

I Methylene blue				
Concentration of methylene blue	1/100	1/500	1/2500	1/12 500
Concentration potential mv	−42	−12	+23	+32
II Quinine*				
Concentration of quinine	1/10	1/100	1/1000	1/10 000
Concentration potential mv	−14	−2	+3 5	+5

* These membranes have not been completely dried

that the limiting reversing concentration of quinine and methylene blue for membranes with low concentration effects was found to be decidedly, sometimes several orders of magnitude, lower than that for Kollodium Schering Kahlbaum DAB6   Table VIII gives two examples, though the results with this brand of collodion are not reproducible in a strictly quantitative way

Asymmetric Membranes

Apart from the well known concentration potentials and chemical potentials due to a different behavior of a surface towards different ions, living cells and tissues sometimes show a potential difference even when they are in contact with two identical solutions   Oster-hout and coworkers for instance have shown (*cf* 24) that across the protoplasm of *Valonia* or *Nitella* a potential difference exists, when both the outer and the inner surface are in contact with cell sap   In this case, since the leads are symmetrical, the asymmetry must lie in the investigated object itself and must be due to a different behavior of the two surfaces towards the same ions   We may call this kind of potential, asymmetry potential

The models, by which chemical and concentration potentials could be imitated, were mainly porous membranes and water immiscible "oil phases "   The underlying difference in behavior of the membrane towards different ions was interpreted as different relative ion mobility in the former and as different solubility in the latter case, having as a consequence in common a higher permeability for either cations or anions   In that respect the greatest differences existed between the dried collodion membrane and the base impregnated collodion membrane among the first group and between basic oils and acid oils among the second group

Asymmetric systems have been less investigated   Cremer's chain of nitrobenzene (*cf* 2) with and without picric acid has been such an asymmetric system   Later Beutner (2, 3) has built up asymmetric chains using different oil phases   The potential differences obtained in these chains are about 100 to 150 mv   Mond (22) obtained potentials of about 20 mv combining two phases of a protein, one on the acid, the other on the basic side of its isoelectric point   Membranes have not yet been used for this purpose   The possibility of obtaining membranes of nearly maximal concentration effect of both signs, *i e* nearly exclusively cation or anion permeable membranes, enabled us to build up asymmetric membrane systems of high efficiency

If an ordinary dry collodion membrane of Kollodium Schering-Kahlbaum DAB6 is combined with an impregnated collodion membrane, to form a double membrane, thus one side being predominantly

cation permeable, the other predominantly anion permeable, a membrane is obtained which is indeed highly asymmetric and yields an asymmetry potential of several hundred millivolts, as is shown in Table IX

To obtain such a membrane certain precautions must be taken. Both membranes must be entirely dry before being combined. They are stuck together by means of a collodion solution, which previously had been allowed to dry to such an extent that on the surface a thin skin was formed. To render this combining layer as thin as possible the membranes are tightly pressed against each other and then allowed to dry. If these precautions are not taken, a diffusion of the dyestuff or alkaloid into the cation permeable membrane takes place which reverses the charge of this membrane, so that a more or less symmetric anion permeable membrane is obtained

TABLE IX

Asymmetry Potentials of Double Membranes in $N/1000$ KCl

Membrane composed of		Asymmetry potential
Inside	Outside	
		$m\gamma$
Quinine-collodion	Collodion	240
Quinine collodion	Collodion	258
Quinine collodion	Collodion	252
Quinine-collodion	Collodion	214
Methylene blue-collodion	Collodion	330
Safranine-collodion	Collodion	320

That such a membrane is asymmetric in the assumed way, one side being cation permeable, the other anion permeable, can be shown by determination of the concentration potentials and the chemical potentials on both sides

For that purpose at first the potential difference of the membrane between two identical electrolyte solutions is measured. Then Solution I is kept constant and the concentration of Solution II is changed. The potential difference is measured again, the difference between the two measurements being the concentration potential of Side II. Correspondingly the concentration potential of the other side is obtained. The concentration potentials thus measured are not entirely independent of the concentration of the constant solution, the variations however are small as compared to the values of the concentration potentials. Table X shows such an experiment. The concentration potentials have the signs expected,

that of the collodion side being positive, that of the quinine side negative   Table XI contains the asymmetry potentials and both concentration potentials of several

TABLE X

Measurement of the concentration potentials of the two sides of a double membrane of collodion outside and quinine-collodion inside   The sign is that of the outside solution, the sign of the concentration potential is that of the dilute solution (both signs refer to the external circuit)

Solution		Measured potential
Inside	Outside	
		mv
(a) KCl N/1000	KCl N/1000	+251
(b) KCl N/100	KCl N/1000	+193
(c) KCl N/100	KCl N/100	+163
(d) KCl N/1000	KCl N/100	+215

Calculated therefrom

Concentration potential outside $b - c = +30$

$a - d = +36$

Average +33

Inside $c - d = -52$

$b - a = -58$

Average −55

TABLE XI

Concentration Potentials and Asymmetry Potentials of Double Membranes
(Signs As in Table X)

Membrane composed of		Concentration potential			Asymmetry potential in KCl N/1000
Inside	Outside	Inside	Outside	Difference	
		mv	*mv*	*mv*	*mv*
Methylene blue-collodion	Collodion	−51 5	+54 5	106	285
Safranine-collodion	Collodion	−56	+49	105	281
Quinine-collodion	Collodion	−48	+52	100	252
Quinine-collodion	Collodion	−55	+33	88	251
Quinine-collodion	Collodion	−46	+30	76	217
Quinine-collodion	Collodion	−36	+40	76	214

membranes   It shows that the asymmetry potential increases as the difference of the concentration potentials increases, in other words the greater the difference of ion permeability on both sides, the higher is the asymmetry potential

To show the same by means of the chemical potentials the same procedure is followed, changing instead of the concentration of one solution, its anions or cations Table XII shows that on the quinine side only the changing of anions affects the potential difference considerably, on the pure collodion side only the changing of cations

One might therefore suppose the mechanism of the asymmetry potentials to be as follows on the anion permeable side anions tend to enter the membrane, which renders the solution there positive in the external circuit, on the other side cations, rendering the solution negative Both effects add up, and lead to a high asymmetry poten

TABLE XII

Chemical potentials of the two sides of a double membrane, consisting of a collodion membrane outside and a quinine collodion membrane inside (The sign is that of the outer solution in the external circuit)

Solution		Measured potential
Inside N/10	Outside N/10	
		mv
KCl	KCl	+57
NaCl	KCl	+61
NaCl	NaCl	+86
KCl	NaCl	+82
KCl	K₂SO₄	+58
K₂SO₄	KCl	+86

Hence	Inside	Outside
Anion effect	29	1
Cation effect	4	25
(Concentration potential)	−46	+30)

tial, positive on the anion permeable side That this cannot be true follows from two facts (1) The observed asymmetry potentials are always positive on the cation permeable side, and (2) they increase as the concentration of the solution decreases, while the opposite should be the case according to the assumed mechanism The highest potential differences were measured in distilled water, they amounted to 450 mv

Fig 3 shows the asymmetry potentials of several membranes in KCl solution of different concentrations (The lower three are asymmetric single membranes)

Apparently the asymmetry potential is not determined by the tendency of the outside ions to enter the membrane, but by the tendency of the inside ions to leave it   The membranes always contain ions of the substance used for the impregnation   This mechanism
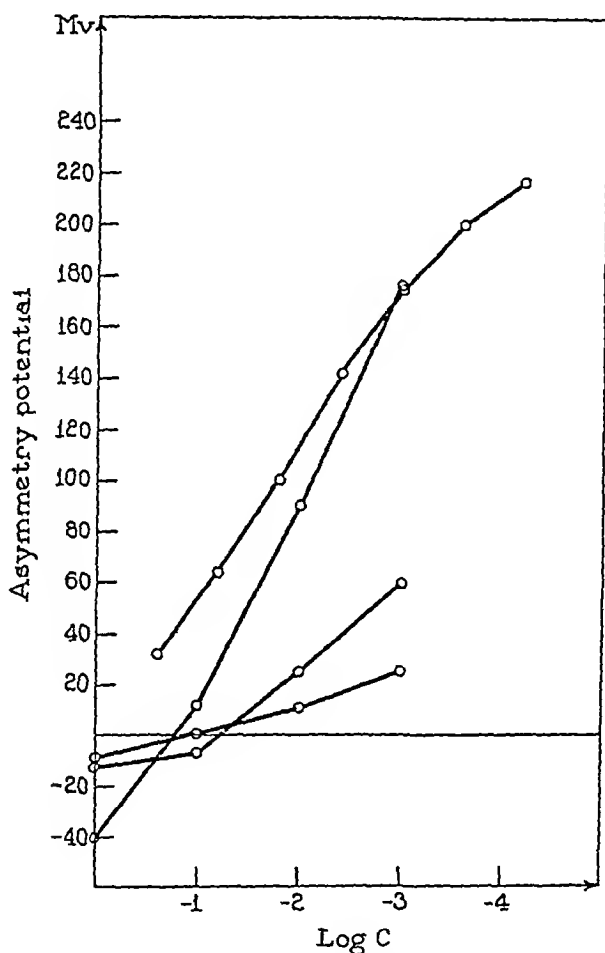
FIG 3 Asymmetry potentials in various concentrations of KCl

must render the solution on the cation permeable side positive, as observed

The effect of the outside ions then must be a lowering of the asymmetry potential, due to their tendency to enter the membrane   Thus the decrease of the asymmetry potential with increasing concentration of the solution can be understood

Cations as well as anions must influence this lowering effect, the cations on the cation permeable side, the anions on the anion permeable side   The lowering of the asymmetry potential by a given electrolyte solution must therefore depend on the electromotive effectiveness of both its cations and its anions   This is the case, as is shown in Table XIII, which contains asymmetry potentials in different electrolyte solutions and for comparison the electromotive effect of their cations at the dried collodion membrane and of their anions at the rhodamine collodion membrane (taken from the paper of Mond)

TABLE XIII

Influence of cations and anions on the asymmetry potential of a double membrane of collodion outside and quinine-collodion inside   The signs refer to the outside solution in the external circuit

Potentials of the ordinary collodion membrane in N/10 solutions of			Asymmetry potentials of the double membrane in N/10 solutions		Potentials of the rhodamine-collodion membrane in N/10 solutions of		
Inside	Outside	mv	of	mv	Inside	Outside	mv
			H₂O	+125			
KCl	KCl	0	KCl	+22			
KCl	NaCl	+48	NaCl	+44			
KCl	LiCl	+74	LiCl	+65			
			K₂SO₄	+67	NaCl	Na₂SO₄	−38
			KCl	+22	NaCl	NaCl	0
			KNO₃	+7	NaCl	NaNO₃	+51
			KCNS	+8	NaCl	NaCNS	+60

It may be noted that it is also possible to obtain an asymmetric double membrane by combining a cellulose acetate membrane and a collodion membrane   These membranes, which yielded comparatively low asymmetry potentials, have not been closely investigated

Asymmetry Potentials in Simple Dyed Collodion Membranes

During the investigation of the reversing effect of basic dyestuffs and alkaloids on the charge of the collodion membrane the observation was made that the impregnated membranes sometimes showed an asymmetry potential of considerable amount, up to 180 mv   in N/1000 KCl and 270 mv   in distilled water   The mechanism of these poten

tials is the same as that of the potentials of the double membranes just described   Table XIV for instance shows that at all asymmetric membranes the concentration effect of the two sides is different and that the magnitude of the asymmetry potential depends roughly on

TABLE XIV

Concentration potentials of both sides and asymmetry potentials of simple dyed membranes (signs as in Table X)

Membrane No	Dyestuff	Concentration potential			Asymmetry potential in KCl n/1000
		Outside	Inside	Difference	
		mv	mv	mv	mv
33	Thionine	−47 5	+36 5	−84	−185
40	Thionine	−33 5	+56 5	−90	−156
30	Thionine	+39	−14	+53	+110
43	Thionine	−10 5	−41 5	+31	+101
47	Brilliant kresyl blue	+14 5	−20 5	+35	+75
29	Methylene blue	+16	−8	+24	+57
26	Basic fuchsin	+41	+29	+12	+48
34	Methylene blue	+2 5	−22 5	+25	+37
27	Quinidine chloride	−6 5	−23 5	+17	+30

TABLE XV

Concentration potentials and effects of anions and cations on the two sides of an asymmetric simple dyed membrane   (Signs as in Table X, anion effect and cation effect obtained as in Table XII)

Membrane No	Dyestuff	Outside			Inside		
		Con centr potential	Cation effect	Anion effect	Con centr potential	Cation effect	Anion effect
46	Toluidin blue	+49	28 5	8	+14	13 5	14
25	Thionine	+8 5	5	11	+45 5	30	3
55	Nile blue-sulfate	+20	12	23 5	−17	3	88 5
54	Safranine	−0 5	4	66	−23 5	3	100
66	Quinine	−44	0	75	−43	1	70 5

the algebraic difference of the concentration potentials   Table XV shows that on a side of the membrane which has a negative concentration potential, anions affect the potential difference more than cations, and on a side with high positive concentration effect the opposite is the case   (That the reversing point is not the concentration

potential 0, but about +20, is due to the fact that the SO_4 Cl effect as such is higher than the K-Na effect)

Thus these membranes behave as though they were composed of two parts which differ in their ion permeabilities   The following reasons are in favor of the assumption that it is only the two surfaces or very thin surface layers whose difference in ion permeability is measured here   A cross section of an asymmetric membrane, impregnated with thionine, was examined under the microscope   No difference in the concentration of dyestuff on the two sides of the membrane could be seen   The thickness of the cation permeable layer on one side, which must be supposed to contain less dyestuff, must therefore be below the limit of microscopic visibility   Furthermore, if the cation permeable side of an asymmetric membrane is scraped with sand paper, a very thin layer thus being removed, the concentration potential of this side becomes negative   At the same time the asymmetry potential changes in the direction expected, sometimes by 100-150 mv , and even changing in sign   Apparently the behavior of the membrane is determined by thin layers at the surfaces   This is no contradiction to the assumption that electrically charged pores determine the potentials   A very thin layer containing pores can determine the transfer number in a transfer experiment, and hence can determine the magnitude of a diffusion potential in a diffusion experiment

To account for the formation of a superficial layer containing less dyestuff than the rest of the membrane, the most probable assumption seems to be the following   The membrane is a solid solution of the dyestuff in collodion, in which, if the membrane is kept in water, a steep diffusion gradient of dyestuff is established at both surfaces, due to the water solubility of the dyestuff   This steep gradient, as the membrane loses dyestuff into the solution, slowly recedes into the interior of the membrane   rendering its surface more and more free from dyestuff and therefore cation permeable   In favor of this assumption is the fact that the asymmetry potential of a dyed membrane, kept in water, mostly changed with time, while in air it kept constant   Furthermore, in water both concentration potentials of such a membrane grow more positive with time, as would follow from the assumed mechanism   Fig 4 shows the change of concentration potentials and asymmetry potentials with time of a membrane   Both

concentration potentials grow steadily more positive, but since the changes are not parallel, the asymmetry potential varies with their difference

How the asymmetry arises, which sometimes is observed on the very first day, is not quite clear, especially since it does not occur regularly
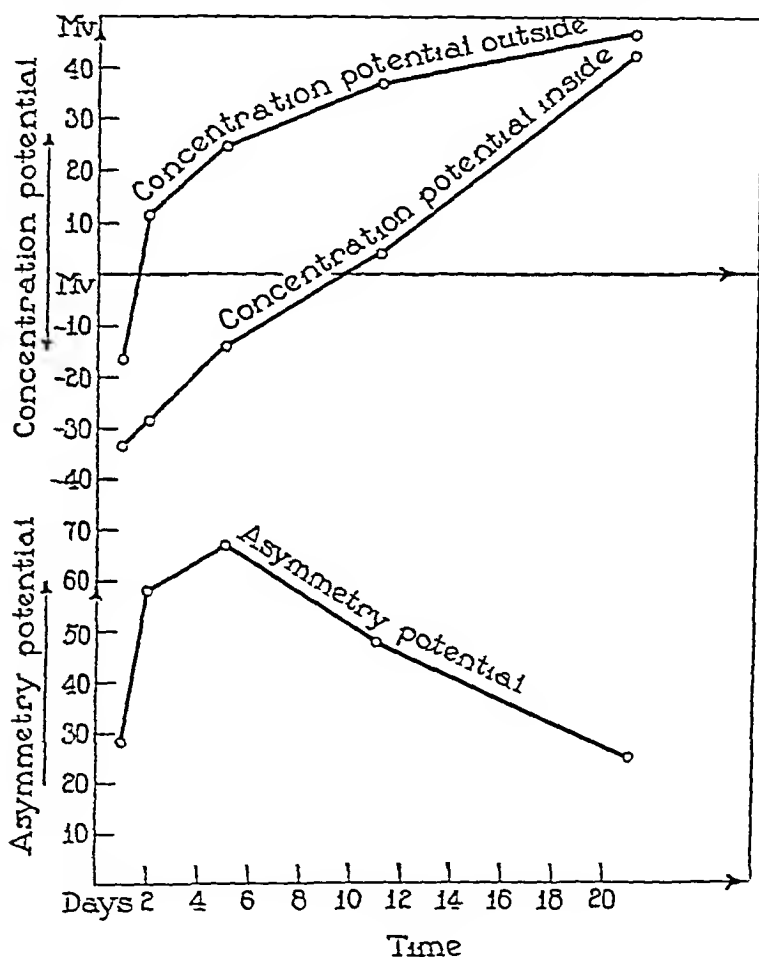
FIG 4 Change of the two concentration potentials and of the asymmetry potential of a simple membrane with time

Sometimes the membranes are highly asymmetric on the first day, sometimes they are symmetric   The drying process of the membrane on mercury is certainly an asymmetric process, one side of the membrane being in touch with air the other with mercury, so that a difference of the two sides is comprehensible   The mercury side behaves

indeed regularly as if it contained more dyestuff, it loses more dyestuff into the water in the first days and it is the more anion permeable side On the other hand membranes which were poured symmetrically on gauze and dried hanging in the air were highly asymmetric too Apparently differences in the drying conditions play a rôle here which cannot be controlled without special precautions

The reason for discussing the asymmetry potentials of simple membranes in detail, though they are not different in principle from those of double membranes, is that they can be very disturbing when working with such membranes and a knowledge of these asymmetry potentials seems valuable, therefore Measurements on dyed collodion membranes should be carried out in duplicate with solutions exchanged and the ion permeability of the membranes should be tested from time to time

DISCUSSION

The points of view to be gained from the results obtained for an analysis of biological asymmetry potentials seem to be the following

The asymmetry potentials here described are due to differences of two surfaces in the behavior towards ions, which may be regarded under the heading of predominant anion or cation permeability A quantitative measure of this property is the concentration effect, the value of which can vary between the thermodynamic limits of -56 mv (exclusive anion permeability) and $+56$ mv (exclusive cation permeability) Now the analysis of an asymmetry potential as to the underlying differences in ion permeability of the two surfaces gives a means to get closer information on the micro structure of cells and tissues Let us consider for instance the following three asymmetric structures of a system with two surfaces (a) One side is more anion permeable, the other more cation permeable, i e one concentration effect is negative, the other positive, (b) Both are more anion permeable, but to a different extent, (c) Both are more cation permeable, but to a different extent All three systems may yield the same asymmetry potential, provided the algebraic differences of the two concentration potentials are the same Thus a closer investigation of an asymmetric system as to the properties of the two surfaces may lead to information about the underlying micro structure

The unusual magnitude of the observed asymmetry potentials as

compared to the potentials otherwise observed with collodion membranes raises the further question whether similar asymmetries play a rôle for the transport functions of epithelium cells, especially in cases where a transport takes place against a high thermodynamic potential The formation of HCl in the stomach epithelium for instance has to overcome a concentration potential of 250–300 mv

SUMMARY

The attempt is made to reconcile the two existing theories on the mechanism of selective ion permeability of the cell membrane by taking into account the molecular arrangement in the substance of the membrane   It is shown that the pore theory and the solubility theory are not contradictory, but two aspects of the same problem, one from the thermodynamic point of view, the other from the point of view of molecular theory

The dried collodion membrane is used as a model in these studies Its different behavior towards anions and cations is explained on the ground of a quasi-crystalline structure of collodion, the NO_3-groups acting as dipoles with the negative charge directed towards the intermolecular spaces, no matter whether these pores are of molecular dimensions or larger   In this way a continuity in the behavior of the ordinary large pored collodion membrane and the dried membrane is established, both theoretically and experimentally   Experiments, with membranes of other cellulose derivatives agree with the mechanism suggested   Membranes of cellophane and ethyl cellulose are negatively charged, membranes of cellulose acetate positively

From solutions of collodion mixed with basic dyestuffs or alkaloids membranes can be obtained more permeable to anions than to cations in contrast to the ordinary collodion membrane   Membranes can be built which give high potential differences even between two identical electrolyte solutions   The asymmetry lies here within the membrane and is artificially produced by gluing together an ordinary collodion membrane with one previously impregnated with a basic dyestuff or an alkaloid

out, for his ever ready stimulation and help   Also I received much
help from discussions with Dr J F Danielli

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THE KINETICS OF PENETRATION

XI. ENTRANCE OF POTASSIUM INTO NITELLA

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INTRODUCTION

In a previous paper[1] the accumulation of potassium by *Nitella flexilis* Ag. was considered briefly. In the present paper the results are presented in detail.

Technique

The cells immediately after collection were washed free of mud and pond water in tap water and were transferred to Solution A[2] in the cold room for a day or so, after which they were separated into strings of from 3 to 10 cells from which the leaflets and nodes were removed. The strings were then left in Solution A in the cold for a few days to 2 weeks, the dead cells being removed at intervals in order to reduce contamination.

In most experiments the cells were transferred to distilled water 1 week to 24 hours before the start of the experiment. They were then placed in the experimental solutions after a rapid rinse in distilled water. In most cases the solutions were contained in 10 gallon oval shaped white enamelled baths,[3] which, except in the cases where the cells were to be kept in the dark, were arranged symmetrically under a bank of six 150 watt lights. By means of a 'Weston photronic' light meter the lamps and pans were arranged so that all solutions were illuminated equally at equal depths.[4] Although it was subsequently found that the illumination was not critical, we continued the above arrangement since it served to keep the temperature of the solutions rather uniformly at about 20 C., a few degrees above the temperature of the cold room in which the experiments were carried out.

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[1] Jacques, A. G., and Osterhout, W. J. V. *Proc. Soc. Exp. Biol. and Med.*, 1934, **31**, 1121.

[2] For composition of Solution A *cf.* Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.* 1933–34, **17**, 87.

[3] In early experiments a larger number of shallow enamelled pans were used.

[4] At the level of the middle of the pans the illumination was about 165 foot candles.

To cut down evaporation the pans were covered with glass sheets   About 2000 cells of a total volume of 25 cc were placed in each pan in 16 liters of solution [5]   At intervals groups of cells were removed and the sap was extracted and analyzed for potassium, and in a few cases the pH was determined

In her studies of the penetration of dyes into *Nitella* Irwin successfully used the method of extracting the sap from individual cells by hand   But owing to the comparatively large amount of sap required, the method was inapplicable here [6] Hoagland and his coworkers[7] also used the method of individual extraction in some of their experiments on the penetration of bromide into *Nitella clavata* These cells, however, are brittle, and as the authors describe it break on bending and expel a drop of sap   In the case of *Nitella flexilis*, it is necessary to cut each individual cell and if the full amount of sap is to be obtained to squeeze Conducting this operation in an atmosphere saturated with water vapor, to avoid evaporation, is tedious, uncomfortable, and costly in time, and it was therefore considered impractical   Instead the sap was obtained in the following way   The strings of cells were removed singly from the experimental solution to distilled water, the dead ones being discarded in the process   After a second rinsing in more distilled water, 20 to 30 strings were quickly laid out on a dry piece of gauze, and were blotted with a second piece of gauze [8]   The strings were then gathered up quickly, rolled into a loose coil and inserted in a dry, 15 ml centrifuge tube by the aid of a glass rod   This was repeated until a sufficient number of cells to yield about 2 ml of sap had been collected

In the early stages of the work, the cells were killed by adding a drop or two of ether and the sap was then expressed by centrifugalizing for a long time at high speed   In this way a sample comparatively free of organic matter was obtained

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[5] Assuming that the sap is 0 1 M with respect to potassium, it follows that if every cell should die and its contents pass into the external solution, the maximum effect would be to increase its concentration by 0 0001 M   But the mortality did not exceed 10 per cent so that the contamination must have been correspondingly less, i e , about 2 per cent at the most where the outside concentration was 0 001 M Actually in one experiment, where the external solutions were analyzed at the end of the experiment, it was found that the concentration, nominally 0 00100 M at the start, had risen to 0 00103 M

[6] In the course of the measurements of volume several hundred individual cells were measured   It was found that the average diameter of the cells used by us was 0 05 cm and the average length 6 cm   Considering them as cylinders, this means that the average volume was 0 012 cc so that if this were all sap and it could be extracted quantitatively by hand, it would still require about 200 cells to yield 2 cc of sap   Actually it was found necessary to use about twice this number of cells for each sample, in our method, which did not utilize all the sap in each cell

[7] Hoagland, D R , Hibbard, P L , and Davis, A R , *J Gen Physiol* , 1926–27, **10**, 121

[8] Both gauzes had, of course, previously been rinsed in distilled water until they were electrolyte-free

But the yield was rather poor, and later it was found that time and cells could be saved by cutting the cells by means of a sharp edged glass rod, and pressing them down in the centrifuge tube The sap which at this point has a great deal of organic matter including chloroplasts, and a gummy substance in suspension can then be centrifuged reasonably free of organic matter in a short time

This method of procedure is obviously subject to errors due, (a) to the contamination of the sap by the liquid in the cellulose wall but not actually in the vacuole or in the protoplasm, and by liquid adhering to the cells, (b) errors due to the evaporation from the vacuole Since the cell wall is thin and extensive, these errors might become serious unless they were guarded against

In order to deal with (a) the cells were rinsed in distilled water twice as described above so as to ensure that the contamination should be dilution only, and in order to reduce this the cells were blotted

TABLE I

Lot	K concentration	Δ from average
	M	
1	0 04550	−0 00055
2	0 04534	−0 00071
3	0 04656	+0 00051
4	0 04496	−0 00109
5	0 04491	−0 00114
6	0 04900	+0 00295
	Av 0 04605	Av 0 00116

In order to guard against (b) the cells were handled as quickly as possible, only a small number being handled at one time and the centrifuge tube was kept tightly capped except when it was necessary to insert cells or to break up and press down those already inserted

In spite of these precautions undoubtedly the sap analyzed had not exactly the composition of the cell sap However by proceeding as far as possible always in the same way it was hoped that the deviation of the extracted sap from the true cell sap would be sufficiently constant to allow definite conclusions

In order to assure ourselves of the validity of the method, 6 lots of cells from the same collection were extracted as described above and the samples were then analyzed for potassium The results are given in Table I

The probable error of the mean is ±0 00044 or ±0 95 per cent of the mean

These results indicate that the method of handling the cells is capable of yielding results sufficiently accurate for biological experiments [9]

An estimate of the relationship of the observed concentration (which is admittedly low due to dilution) to the true concentration has been made in a separate

[9] If the last observation in this series is rejected the agreement is much better, the probable error of the mean being then ±0 00034 or ±0 75 per cent of the mean

experiment in which the cells were divided into two groups   From one group the sap was extracted by crushing as explained above, while in the other it was extracted by cutting and squeezing the sap out of each individual cell   This was done in a water-vapor saturated atmosphere maintained in a glass-covered box, of about 3 cubic feet capacity   The atmosphere was kept saturated by passing in steam at atmospheric pressure, and was checked by means of a hair hygrometer   Rubber sleeves were permanently attached to the box through which the hands were thrust into the interior of the box

The following results were obtained   Concentration of sap extracted by crushing, 0 04916 M, by hand squeezing, 0 05800 M   Since the maintenance of the saturated atmosphere is not easy this dilution of 15 3 per cent is to be regarded as a maximum figure   If this dilution is not subject to much variation it will have only a negligible influence on the results which are always comparative That this is the case is shown by the fact that the results are reproducible to a satisfactory degree

After centrifugalization, 0 5 cc samples of sap were measured out by means of washout pipettes[10] into micro platinum crucibles of 2 ml capacity   These were taken to dryness on the water bath, dried in the oven, and ignited just below red heat, to white ash

For potassium we have worked out a microgravimetric method, based on the Lindo-Gladding modification of the chloroplatinate method,[11] using the filter stick and micro beaker technique of Emich [12]

The potassium method was tested out on a synthetic sap having about the same composition as the sap of *Nitella clavata* according to the analyses of Hoagland [7]   The found amounts compared with the actual amounts present are as follows

Actual concentration	Found concentration		
K molar	K molar	Δ	Per cent Δ
0 04956	0 04946*	—0 0010	—0 23
	0 04980	+0 0024	+0 49
Average deviation			±0 36

* In general the average weight of the chloroplatinate salt was between 5 and 10 mg   And inasmuch as with the micro balance it is possible to weigh to 5 γ with ordinary precautions, the method is clearly capable of yielding results more precise than the micro methods based on cobalti-nitrite precipitation which are so widely used

[10] According to Pregl, F , Quantitative organic microanalysis, Philadelphia, Blakiston, 2nd English edition, 1930, 118

[11] We are indebted to Prof Benedetti-Pichler for advice and help in connection with the microanalyses

[12] Emich, F , Lehrbuch der Mikrochemie, Munich, Bergmann, 2nd edition, 1926, 84–88

Volume Measurement

In the case of *Valonia* which is nearly in osmotic equilibrium with the sea water, the entrance of electrolytes already present is made clear only when the increase in volume is determined as well as the electrolyte concentration, since the cell takes in water and expands at such a rate as to keep the electrolyte concentration nearly constant   It was therefore necessary to discover whether in these experiments with *Nitella* an increase in volume would occur   A separate investigation of this point was therefore undertaken

In these experiments a number of groups of cells were treated in various ways approximating the experimental conditions later applied, and the volume change was measured   Since each cell is approximately a cylinder, the volume was measured by determining the average diameter and the length

In order to do this the cells of each group were laid out in parallel shallow grooves cut in the surface of a layer of paraffin wax cast in a covered Pyrex dish

In order to prevent the cells from altering their positions in the grooves they were confined lightly by means of glass strips cut from microscope slides laid over the grooves but exerting very slight pressure   Solutions similar to those used subsequently in the experiments were then introduced to the depth of about 1½ inches over the cells   In order to reduce changes in the pH due to cell metabolism the solutions were changed frequently   The diameter was measured by bringing a microscope over the cell and determining the diameter at several points, by means of a Zeiss travelling ocular micrometer   The length was determined by photographing at the prescribed times, and measuring the length of the cell on the plate by means of a Starrett vernier caliper reading to 0 005 cm   This procedure was used because the ends of the cells are not parallel plane surfaces so that it was necessary to be sure that the measurements were made between the same two points   This could be done by means of the photographs which could be measured all at one time although taken several days apart   In the length photographs the magnification factor was about 1 75 and in order to reduce all measurements to the same terms an unchanging reference of length and diameter was provided   This consisted of a length of mechanical pencil "lead" which because of its method of manufacture, extrusion under great pressure through a die is very uniform in diameter

No volume data will be given since it was found that the cells did not change in volume by more than the experimental error   Duplicate measurements on a single cell indicated that the radius could be determined to about 0 4 per cent and the length to about 0 5 per cent.[13]

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[13] Since the volume of a cylinder is given by the expression $V = \pi r^2 l$ the error of the volume due to the error in the measurement of l would also be $\pm 0 5$ The error in V due to the error in the measurement of r can be found by solving the partial differential

$$\frac{\partial V}{\partial r} dr = 2 \pi r l dr$$

The External Solutions

Except where otherwise stated, the solutions to which the cells were exposed were prepared by mixing suitable amounts of potassium chloride, potassium bicarbonate, and calcium chloride solutions with distilled water, and bringing them into equilibrium with the CO_2 of the air by prolonged aeration   The pH thus depended on a CO_2 bicarbonate system   Since the former was fixed the various pH's were secured by altering the amount of the latter

RESULTS

Illumination —In view of the findings of Hoagland, Hibbard, and Davis[7] that illumination had an important effect on the accumulation of the bromide chloride, and nitrate ions in the case of *Nitella clavata*, it seemed desirable to determine if the entrance of potassium into *Nitella flexilis* is also dependent on light   In the two experiments performed, the cells were exposed to external solutions having the following composition, KCl 0 000900 M, $KHCO_3$ 0 000100 M, $CaCl_2$ 0 000025 M   The initial pH was 7   This changed slightly as indicated in Table II   The experiment was performed in a room which at the start had a temperature of 17°C   Owing to the heating effect of the illuminating system, this rose during the experiment   These changes are also recorded in Table II   The results are illustrated by Fig 1

It is seen that the increase in the concentration of potassium in the dark in 96 hours is 40 7 per cent, and in the light 37 2 per cent   It appears therefore that the rate of entrance of potassium does not depend on illumination   This is perhaps the more surprising in view of the fact that in the light the pH rose, indicating considerable photosynthetic activity   It is perhaps worth while mentioning also that in these experiments there was no nitrate ion in the external solution, so that the mechanism of entrance based on nitrate transformation to protein discussed in a previous paper cannot apply here

The Influence of External pH —It has been shown that the penetration of K^+ into *Valonia macrophysa* is markedly affected by the external pH, in spite of the fact that the cell tends by means of its metab-

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To do this the average values for $r = 0$ 025 cm and $l = 6$ 0 cm were used   Whence it appears that the error due to r where $V = 0$ 01177 is 0 000094 or nearly ± 1 per cent   The total error therefore in volume measurement may have been ± 1 5 per cent

olism to nullify artificial changes in the pH of the sea water   In the dark, the cells fail to grow and under these conditions the intake of K ceases, and when the pH is lowered to the point where $(K)(OH_i) > (K_o)(OH_o)$ there is a slow replacement of K^+ inside by Na^+

TABLE II

Time	Illumination	Temperature of external solution	pH of external solution	K concentration	pH of sap
hrs		C		u	
0	Light	21 0	6 80	0 04359	6 05
0	Dark	20 7	6 90	0 04359	6 05
24	Light	20 4	7 45	0 04854	6 05
24	Dark	20 7	—	0 04896	6 00
48	Light	18 9	7 42	0 05454	5 80
48	Dark	19 4	6 60	0 05547	5 85
96	Light	21 1	7 20	0 05978	5 95
96	Dark	19 5	6 80	0 06131	5 85
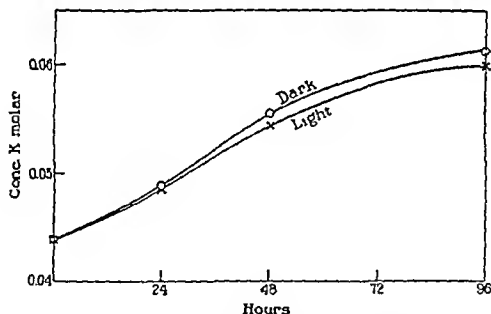
FIG 1   Time curves showing the penetration of potassium into *Nitella*, in the light and in the dark   The curves are drawn free hand to give an approximate fit   this applies to all the figures

Similar experiments have been carried out with *Nitella* using the technique described earlier in this paper   In these experiments the pH was maintained by means of a bicarbonate CO system   The solutions were initially in equilibrium with the CO_2 and the proportion of bicar-

bonate ion added was varied   The concentration of $CaCl_2$ was uniformly 0 0005 M

Figs 2 to 5 illustrate quite clearly that the rate of entrance of potassium at any external concentration from 0 00010 M to 0 0100 M does not depend on the external pH at least between pH's 6 and 8   It is
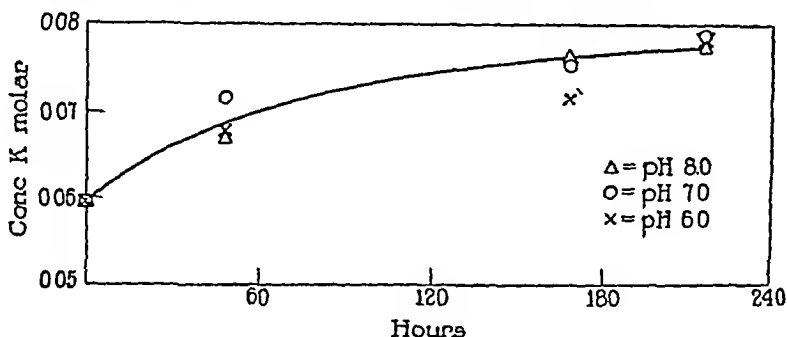
FIG 2 Time curves showing the penetration of potassium into *Nitella* in the dark at different external pH values, the external concentration of potassium being 0 00100 M in all cases
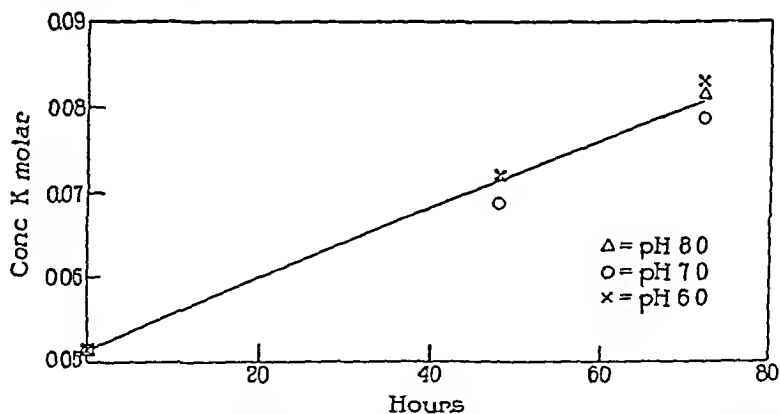
FIG 3 Time curves showing the penetration of potassium into *Nitella* in the dark at different external pH values, the external concentration of potassium being 0 0100 M in all cases

necessary to point out that no conclusions should be drawn from these experiments as to the effect of external concentrations of K^+ on the rate of entrance of potassium, since cells from different collections, not necessarily reacting exactly alike, were employed in different experiments   But this does not apply to the effects of pH since in each experiment where the pH was varied the cells were from the same lot

External Concentration of Potassium —In the case of *Valonia* it has been shown that the rate of gain of potassium moles at the same pH increases with increasing concentration of potassium in the sea water
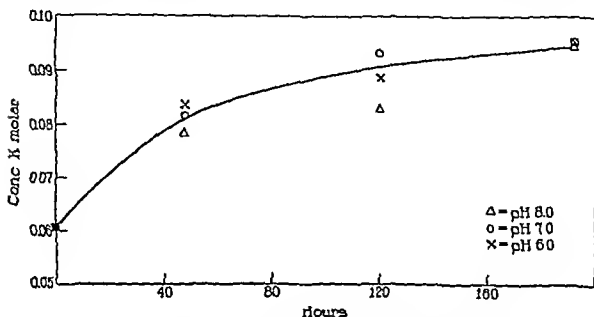
Fig 4 Time curves showing the penetration of potassium into *Nitella* in the light at different external pH values the external concentration of potassium being 0 00100 m in all cases
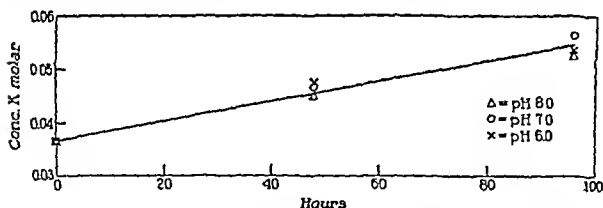
Fig 5 Time curves showing the penetration of potassium into *Nitella* in the light at different external pH values, the external concentration of potassium being 0 00010 m in all cases As the experiments were performed in the light, photosynthesis may have raised the pH just outside the protoplasm to higher values than those here stated

Similar experiments with *Nitella* have now been carried out   In these experiments the concentration of K+ in the external solution was varied from 0 0100 m to 0 00010 m without damage to the cells   *Nitella*

is less sensitive in this respect than *Valonia* with which it was not possible to reduce the potassium content of the sea water by more than 1/2 or to increase it by more than 4 times without injury occurring
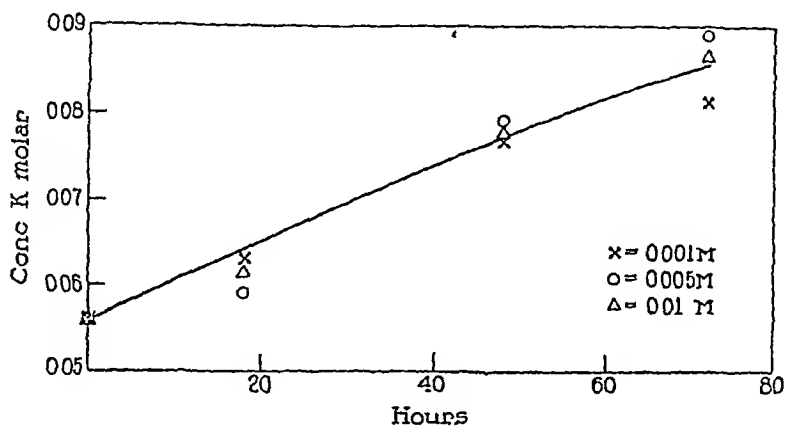
FIG 6 Time curves showing the penetration of potassium into *Nitella* in the light at different external potassium concentrations, the pH being 7 0 in all cases
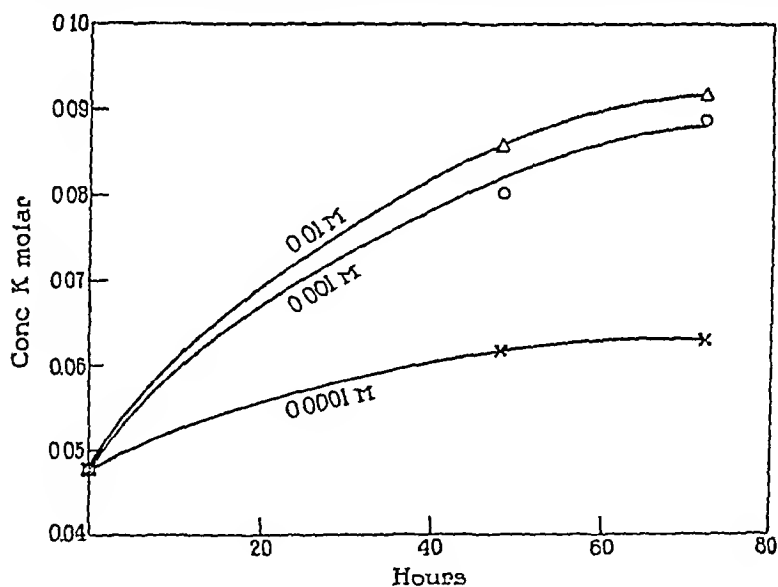
FIG 7 Time curves showing the penetration of potassium into *Nitella* in the light at different external potassium concentrations, the pH being 7 0 in all cases

in a short time   At the start of the experiments with *Nitella* the pH was adjusted to about 7, corresponding to a concentration of about 0 0001 M bicarbonate ion in a solution saturated with CO_2 at atmospheric pressure

The results of the experiments are given in Figs 6 to 9

As the figures show, the external concentration of potassium (between 0 01 M and 0 001 M) has not much effect on the rate of entrance
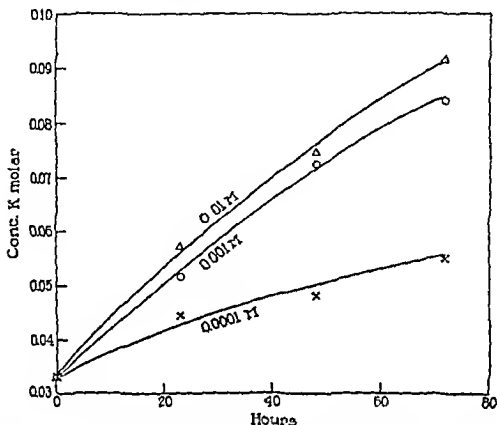
FIG 8 Time curves showing the penetration of potassium into *Nitella* in the light at different external potassium concentrations, the pH being 7 0 in all cases
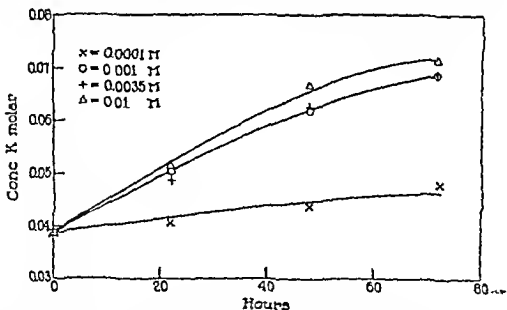
FIG 9 Time curves showing the penetration of potassium into *N* light at different external potassium concentrations, the pH being 7 0 in

But when the concentration is lowered to 0 0001 M, the rate decreases sharply

DISCUSSION

It has been suggested that the entrance of cations into the vacuole of the large single cell of *Valonia*, may depend on the gradient $(K_o) (OH_o) - (K_i) (OH_i)$, where K and OH are the concentrations of the potassium ion and hydroxyl ion respectively, and the subscripts o and i refer to the external solution and the cell sap   However, in the case of *Nitella* it has been shown above that when the external concentration of potassium is as low as 1×10^{-4} molar, and the external OH activity is 1×10^{-8}, K^+ still enters, although the internal concentration of potassium is about $10^{-1\ 3}$ and the internal OH activity is not less than 10^{-9}, so that the product (K) (OH) is higher inside

This is not surprising since the cell has energy at its disposal Our problem is to ascertain how the energy is applied to bring about this result   In the case of *Valonia* we have a hint on account of the effect of external pH and we may set up the working hypothesis that potassium enters chiefly as KOH   In *Nitella* we gain no such hint from the present experiments though it is possible that we may obtain it when lower concentrations are studied

There are, however, other factors which may affect the rate of entrance   It might be suggested that the internal activity of the potassium may differ greatly from the concentration found by analysis However, if it is permissible to apply calculations of Zscheile[14] in the case of *Nitella clavata*, it is clear that the internal ionic strength does not differ sufficiently from that of the external solution to account for the entrance of potassium   For example, the value of $(K_o) (OH_o)$ in the case just quoted is about 10^{-12} so that if $OH_i = 10^{-9}$, K_i may not exceed 1×10^{-3}, hence if the internal concentration is $10^{-1\ 3}$ the activity coefficient of the potassium ion would have to be $10^{-1\ 7}$ to make the products equal

But if the ionic strength is inadequate to account for the low potassium activity, it might be argued that the potassium in the cell may be part of a non-polar compound   This question has been discussed in a previous paper,[15] and it has been concluded that the

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[14] Zscheile, F P , Jr , *Protoplasma*, 1930, **11**, 481
[15] Jacques, A G , *J Gen Physiol*, 1934–35, **18**, 283

possibility of the existence of non polar potassium compounds in nature is extremely remote Since the sap after centrifugalization is somewhat cloudy there remains also the possibility that the potassium may be present as part of an insoluble, but colloidally dispersed compound The arguments in favor of this possibility are, (a) the fact that potassium salts are known[16] which are more soluble in non polar solvents than in water, and that calcium salts of such compounds have actually been isolated from the protoplasm of cabbage leaves and other plants [17] (b) the fact that in the slightly gelatinous sap obtained by extracting each cell by hand the potassium concentration was found to be about 15 per cent greater than in the sap extracted from the same cells by crushing and centrifugalization The difference may possibly be due to dilution, but it might also be argued that from the clarified sap, which is not gelatinous, the insoluble colloidal compound has been precipitated In order to investigate this point further the following experiments were carried out

Samples of sap were obtained by crushing the cells and after permitting the debris, such as chloroplasts, to settle out as much as possible, the gelatinous sap was divided into two portions, one of which was analyzed for potassium at once while the second was ultrafiltered under 3 atmospheres pressure, through a dense collodion filter, and then analyzed for potassium At the same time the rest of the group of cells from which the sap was extracted were transferred from distilled water to 0 001 M potassium salt solution for 3 days At the end of this time the sap was extracted and analyzed raw and ultrafiltered The results are given in Table III

These results indicate clearly that the sap does not contain colloidally dispersed potassium, and hence we may rule out definitely non polar potassium compounds and insoluble potassium salts

It may also be argued that the measured pH of the sap (which in our experiments varied from 5 3 to 6 0) does not represent the true pH at the interface between the sap and the internal surface of the protoplasm However, in the case quoted above, in order to permit the entrance of potassium it would be necessary that the internal pH at the interface be as low as 3 3 This could only happen if the cell were

[16] Hundeshagen, F , *J prakt Chem* 1883, **28**, N s 219

[17] Smith J A. B , and Chibnall A C *Biochem J* , London, 1932 **26**, 1345 Chibnall A C and Channon, N J , *Biochem J* , London, 1929, **23**, 176

capable of producing an acid stronger than H_2CO_3 or if a supersaturated solution of CO_2 could be produced   The latter is improbable since the protoplasm is highly permeable to CO_2 and hence the supersaturation of carbonic acid would be limited by its escape to the external solution   The former possibility, however, cannot be ruled out entirely

It now remains to examine the possibilities that other gradients are more favorable to the entrance of K   The question of entrance in the form of nitrates or carbonates has already been considered in connection with other plants [15]   It was pointed out that entrance as nitrate, while theoretically possible in the case of flowering plants, must be rejected in the case of *Valonia*   In the case of *Nitella* it is also very improbable since no nitrate ion was added to the external solution

TABLE III

Comparison of Potassium Concentrations of Raw and Ultrafiltered Sap

Sap of cells before exposure to 0 001 M K solution		Sap of same cells after 3 days' exposure	
Description of sap	Concentration K	Description of sap	Concentration K
	M		M
Raw	0 0334	Raw	0 0783
Ultrafiltered	0 0330	Ultrafiltered	0 0788

It is possible, however, that the potassium bicarbonate concentration may be sufficient under natural conditions and in the laboratory to set up an inward diffusion of potassium bicarbonate   Thus in our experiments the pH was regulated by the concentration of bicarbonate ion, added as potassium bicarbonate, and the concentration of CO_2, which was in equilibrium with the CO_2 of the atmosphere   In natural pond water in most cases also there is some bicarbonate ion present   But if potassium bicarbonate enters the cell as such, either the pH must increase, and owing to the accumulation of bicarbonate ion the entrance must cease, or else the cell must have methods of disposing of the ion, such as (a) by displacement by a stronger acid anion, or (b) by using the CO_2 in the elaboration of organic compounds, and thus leaving the KOH free to react with another acid, either produced in the cell or brought from the external solution (for example HCl)

In our experiments it was found that while the pH as measured by the glass electrode varied between 5 3 and 6 0 it did not rise appreciably during penetration, at least not in the whole volume of the sap Any bicarbonate ion therefore which penetrated the sap must have been disposed of in some way   It does not seem possible that it could have been used by the cell to build organic compounds, for such a process would presumably require light especially in the formation of carbohydrates   But as it has been shown above, the rate of penetration was unaffected by the illumination   It seems entirely possible, however, that the cell may dispose of bicarbonate ion by means of a stronger acid   But for accumulation to occur it would be necessary that the protoplasm should not be very permeable to the potassium salt formed, since otherwise it would escape too rapidly

If the entrance of potassium bicarbonate depends simply on diffusion through the protoplasm, the rate should be proportional to the external bicarbonate concentration   But as a matter of fact, in our experiments on the pH effect the bicarbonate concentration varied from about 0 00001 M to 0 001 M without affecting the rate   However, there remains the possibility that the rate of entrance of $KHCO_3$ may depend on the rate at which the HCO_3 ion is being disposed of inside

As far as we can see at present the only gradient which may possibly favor the inward flow of potassium is the potassium bicarbonate gradient   Failing this we may assume that in some way, not yet known, the "effective" internal product[18] (K) (OH) is less than the product calculated from the hydrogen ion activity and the potassium concentration

In studies of the penetration of cations in *Nitella clavata*, Hoagland and Davis[19] found that in several cases the cells were able to remove chloride ion so completely from the external solution that no test could be obtained by means of silver nitrate, so if the penetration takes place by simple diffusion of chloride ion, the "effective" internal concentration must be practically zero   If the chloride enters as

[18] Osterhout W J V *Proc Nat Acad Sc* 1935 21, 125

[19] Hoagland D R , and Davis A R *J Gen Physiol* , 1922–23, 5, 629 24, 6, 47   Hoagland D R in *Contributions to* ... versity Stanford University Press 1930, 131   Ho and Hibbard P L *Plant Physiol* , 1928 3, 473

HCl, as is suggested by the fact that the absorption of strong acids (e g HNO$_3$[19]) takes place more readily from acid solution, the "effective" internal product of HCl must also be extremely low, since the exhaustion of the external solution took place even when the external pH was 5 0 to 5 5

The question with regard to entrance of potassium is not yet settled definitely, but the following preliminary experiment shows that in all probability there is an external concentration of potassium at which no potassium goes in   A group of cells was divided into two parts, from one of which the sap was extracted (Group 1) for potassium analysis, and the other was transferred from Solution A to distilled water for 5 days (Group 2)   The sap from a part of this latter group was then analyzed for potassium (Group 2, section *a*), and the rest of the group was then transferred to 0 001 M potassium solution for 3 days, after which the sap was analyzed (Group 2, section *b*)   The results are given in Table IV

Comparing this experiment with the results in 0 0001 M in Figs 8 and 9 it seems that there must exist a concentration between 0 0001 M and 0, at which the potassium neither goes in nor comes out   It is necessary, however, to regard this experiment with caution, since the results may merely mean that the low potassium content is due to the presence of injured cells, in the group kept for 3 days in distilled water Even the subsequent rise in the potassium concentration in the same group of cells, after removal to 0 001 M K solution, is not conclusive, although it does suggest that not much injury has occurred [20]   Provisionally it seems probable that potassium can leave uninjured cells However, it is clear that for some reason, not yet clarified, the passage of potassium from the vacuole to the external solution is impeded much more than the flow in the opposite direction

If we could determine the external activity product at which potassium neither enters nor leaves we might put the effective product inside equal to this at this particular external concentration [18]

It now remains to consider the question of the rate of entrance of

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[20] But, of course, it would be possible to obtain this result if part of the cells were severely injured and low in potassium, provided others had escaped injury and had taken in enough potassium to mask the dilution due to the injured cells

potassium   In the study of models, [1] attention has been drawn to the importance of the unstirred layers which exist at phase boundaries It bas been shown that the rate of transfer of potassium guaiacolate (which may be called KG) between two aqueous phases, separated by a non aqueous layer of HG, depends on the concentrations of the salt in the aqueous phases and the partition coefficients of the salt between the aqueous phases and the non aqueous phase   These determine the concentration gradient of KG The consequences are, (a) that if the salt which diffuses through the non aqueous phase has an anion derived from a weak acid, such as guaiacol, the rate of entrance will be influenced by the external pH, for the pH will determine the product (K) (G), (b) that the rate of entrance should increase if the external

TABLE IV

Description of sample		Concentration K
		M
Group 1	Sap from cells from Solution A	0 0560
Group 2 (a)	Sap from cells after 3 days in distilled water	0 0334
(b)	Sap from cells after 3 days in distilled water and subsequently 3 days in 0 001 M K solution	0 0783

concentration of K^+ is increased, (c) and finally that if the external concentration of the guaiacolate ion is increased the rate of entrance of K^+ is increased

The question arises whether the penetration of potassium into *Nitella* is in any way related to the penetration in guaiacol models The rate in *Nitella* is independent of the pH and of the potassium concentration over a wide range   Moreover in order that there should be an inwardly directed gradient for potassium it is clear that the internal partition coefficient must be very low [*]   However, it seems probable that other factors are involved   In the models, there is a large amount of HG, but in *Nitella* the amount of HX in the outer

[1] Osterhout, W J V , *J Gen Physiol* , 1932–33 16, 529

[*] In the case of the models where the non aqueous phase is guaiacol, the external and internal coefficients cannot differ so much, but in the case of living cells, where there are at least two non aqueous layers, which may be very different very different partition coefficients are possible

non-aqueous surface is limited as compared with K, so that the rate of entrance will be determined chiefly by this limiting factor   This point has been discussed in the case of ammonia concentration[18] and it has been shown that if as a necessary preliminary to penetration a "reversible" reaction $NH_4OH + HX \rightleftharpoons NH_4X + H_2O$ takes place in the non-aqueous surface layer, the rate of penetration must necessarily increase more and more slowly as the external concentration of NH_4OH increases, i e $\dfrac{dr}{dc}$ must decrease (here r is rate of entrance and c is the external concentration) until it practically becomes independent of the NH_4OH concentration   If in the penetration of potassium into *Nitella* a similar reaction, $KOH + HX \rightleftharpoons KX + H_2O$, takes place, a similar diminution in $\dfrac{dr}{dc}$ must occur

This is entirely in keeping with the result we obtained, that the rate of penetration in *Nitella* is independent of the external concentration of potassium except at the lowest concentrations   However, the failure of the pH effect remains to be explained   Thus, as Figs 7 to 9 show, the increase in $\dfrac{dr}{dc}$ appears when the product (K) (OH) is between 10^{-10} and 10^{-11} [23]   But from Fig 5, in which the pH is varied while K remains constant, it appears that even when the product (K) (OH) has fallen to 10^{-12} the rate was not different than when it was 10^{-10} But it must be remembered that as the experiments were done in the light photosynthesis may have raised the pH just outside the protoplasmic surface to a higher value than that here reported

It may be remarked that if potassium penetrates chiefly as KOH its rate of entrance will not continue to increase indefinitely as the pH goes up because above a certain pH secondary changes will ensue [24]   Hence there will be an optimum pH for the entrance of potassium

SUMMARY

The rate of entrance of potassium into *Nitella flexilis* has been investigated, and it has been shown that (*a*) at the concentrations

[23] Assuming that the activity coefficient of KOH is unity
[24] As, for example, in *Nitella* when leached with dilute NaOH (*cf* Osterhout, W J V , and Hill, S E , *J Gen Physiol* , 1933–34, **17**, 99)

studied the rate is independent of the external pH between 6 and 8 but it is possible that at lower concentrations a dependence may be found, (b) that it does not vary much with the external potassium concentration between 0 01 and 0 001 M, but appears to vary more with the potassium concentration below this limit

It has also been shown that the rate is independent of the illumina tion, in contrast with the penetration of halides into *Nitella clavata* studied by Hoagland

It has been found that potassium leaves the cells in distilled water, and since this does not seem to be the result of injury, there is apparently a concentration between 0 and 0 0001 M at which potassium neither enters nor leaves the cell   In *Valonia* increase of external potassium increases the rate of entrance as shown in the increase in moles of potassium in the sap   In *Nitella* this is true below an external concentration of 0 001 M   In *Valonia* this increase is paralleled by the increase in entrance of water so that little or no change in concentration occurs, but in *Nitella* no growth occurred during the experiment and in consequence the concentration of potassium in the sap increased

It has been shown that the potassium content of the raw gelatinous sap is no greater than that of its ultrafiltrate, so that it is not possible to assume that any of the potassium is bound up in the cell in colloidal compounds

It has been pointed out that all the gradients between the sap and the external solution are unfavorable to the entrance of potassium except the potassium bicarbonate gradient   However, on other grounds entrance as potassium bicarbonate is not considered to be very probable

Various modes of entrance are discussed and it has been concluded that the subject must be investigated further before a definite answer can be given

CHEMICAL RESTORATION IN NITELLA

I Ammonia and Some of Its Compounds

By W. J. V. OSTERHOUT

(From the Laboratories of The Rockefeller Institute for Medical Research)

Normal cells of *Nitella* behave very differently toward potassium and sodium   Between a spot in contact with 0 01 M KCl and one in contact with 0 01 M NaCl there may be a P D of 85 mv or more[1] (this is called the potassium effect)   This behavior is completely altered when the cells are kept for 2 or 3 days in distilled water   The cell then loses its power to discriminate between sodium and potassium so that the potassium effect is lacking

The potassium effect may be restored by action currents[2] and by mechanical means[3]   It will be shown in this paper that it can also be restored by chemical means

A typical experiment is as follows[4]   Cells deprived of the potassium effect (by remaining for 6 days in distilled water) were arranged as in Fig 1 with 0 01 M NaCl at all contacts   The substitution of 0 01 M KCl for 0 01 M NaCl at C (at 1 in Fig 2) did not make the P D more

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[1] Osterhout W J V   *J Gen Physiol* 1929–30 **13**, 715

Osterhout W J V and Hill S E   *J Gen Physiol* 1934–35 **18**, 681

[3] Hill S E and Osterhout W J V   *J Gen Physiol* 1934–35 **18**, 687

[4] Similar results were obtained with cells collected in June and kept in Solution A (this contains calcium *cf* Osterhout W J V and Hill S E   *J Gen Physiol* 1933–34 **17**, 87)   These cells (like those found later in summer) showed no potassium effect or irritability   It seems possible that during the season of active growth the substances necessary for these effects are not produced in sufficient quantities, perhaps because the raw materials are used up in the process of growth

The experiments were performed on *Nitella flexilis* Ag   The technique unless otherwise stated is similar to that described in previous papers (*cf* Osterhout W J V and Harris E S   *J Gen Physiol* 1927–28 **11**, 391   Osterhout, W J V and Hill S E , *J Gen Physiol* 1929–30 **13**, 547, 1930–31 **14**, 385, 473 1933–34 **17**, 87   Blinks, L R   *J Gen Physiol* , 1929–30 **13**, 361)
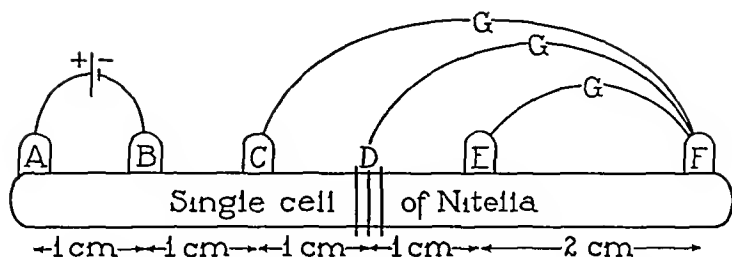
FIG 1   Arrangement for testing cells (placed on a block of paraffin) with various solutions   C and D are flowing contacts for changing solutions during recording All contacts are made through saturated calomel electrodes   GGG represent Cambridge string galvanometers with thermionic amplifiers (the measurement is essentially electrostatic)   The recording instrument was a Cambridge Type A string galvanometer in which the single string had been replaced by three tungsten wires, each 5μ in diameter   Careful calibration shows the deflections to be proportional to the applied voltage within the limits of the recording paper here employed
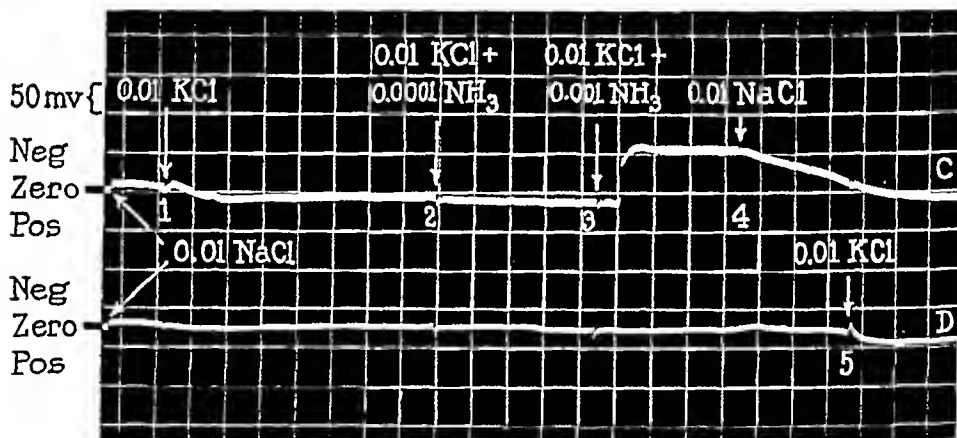
FIG 2   Photographic record of an experiment arranged as in Fig 1   At the start C and D were in contact with 0 01 M NaCl (E was omitted)   During the first part of the experiment all changes were made at C (upper curve)   the fact that D (lower curve) remained unchanged shows that the common contact F was also free from change

At 1, 0 01 M KCl was applied to C with little result, at 2, it was replaced by 0 01 M KCl + 0 0001 M NH₃ which also produced little result   At 3, this was replaced by 0 001 M NH₃ + 0 01 M KCl   This produced a normal potassium effect (about 84 mv)   When 0 01 M NaCl was applied (at 4) the P D   returned to normal   At 5, 0 01 M KCl was applied to D, producing a small increase in positivity, somewhat greater than that seen in the upper curves (at 1)

Temperature about 24°C   The vertical marks are 5 seconds apart   Previous to the experiment the cell was kept in distilled water for 6 days

negative (as it would have done had the potassium effect been present)
At 2 the 0 01 M KCl was replaced[5] by 0 01 M KCl + 0 0001 M NH₃,
but as this had no effect[5] it was replaced (at 3) by 0 01 M KCl +
0 001 M NH₃ This concentration of NH₃ restored the potassium
effect as is shown by the fact that the P D became about 84 mv more
negative[7] When this solution was replaced by 0 01 M NaCl (at 4)
the P.D returned to the normal, showing that no injury had occurred

In many cases the potassium effect was obtained with 0 01 M KCl +
0 0001 M NH₃ Apparently the concentration required depends on
the condition of the cell

Similar results were obtained by employing NH₄Cl (0 0001 M to
0 01 M) and tetraethyl ammonium chloride (0 0001 M to 0 01 M) in
place of NH₃, but they gave a smaller percentage of successes than
NH₃, possibly because the latter penetrates more rapidly (NH₃ appears
to combine with a constituent of the protoplasmic surface in entering[5])

The experiment was then varied as follows Cells which had lost
their potassium effect by leaching in distilled water were tested by
applying 0 01 M KCl at one spot as this gave no result the KCl was
replaced by 0 01 M NaCl Then 0 001 M NH₃ was applied for about
30 seconds After this the application of 0 01 M KCl gave the potas-
sium effect in nearly all cases An application of 0 01 M NaCl was
always made after the KCl to make sure that the negativity was not
the result of injury

[5] NH₃ as here used includes NH₄OH

[6] Substitution of 0 001 M NH₃ for 0 01 M NaCl in living cells produced an effect
(about 17 mv positive) which was somewhat larger in dead cells (about 28 mv)
Substitution of 0 01 M NH₄Cl for 0 01 M NaCl produced a negative change which
varied with the particular lot of cells employed (up to 40 mv) and in dead cells
a very small negative effect (about 4 mv) Substitution of tetraethyl ammonium
chloride 0 01 M for 0 01 M NaCl produced a negative change of about 145 mv but
this was not wholly reversible since after returning to 0 01 M NaCl the spot was
still about 86 mv more negative to 0 01 M NaCl than before (the actual effect,
aside from injury was probably about 58 mv) With dead cells the negative
change was about 16 mv

In all experiments the pH of the NH₄Cl and of the tetraethyl ammonium
chloride was about 5 4

[7] In some experiments the restoration of the potassium effect was slow or in
complete

[8] Osterhout, W J V , *Proc Nat Acad Sc* , 1935, 21, 125

In other cases the cells were first tested for potassium effect and irritability with negative results   Then after soaking the entire cell in NH₃ it was tested for irritability with 0 01 ᴍ NaCl at all contacts   This was replaced at one spot by 0 01 ᴍ KCl or 0 01 ᴍ KCl + 0 001 ᴍ NH₃ which gave a potassium effect   After this 0 01 ᴍ NaCl was applied to see if the ᴘ ᴅ returned to normal   In some cases no potassium effect was observed until a second application of 0 01 ᴍ KCl or 0 01 ᴍ KCl + 0 001 ᴍ NH₃ was made at the same spot [9]

In some cases 0 01 ᴍ NaCl was not employed at certain spots until after the application of KCl

In order to determine whether irritability could be restored it was necessary to treat the entire cell (or a considerable portion of it)   A
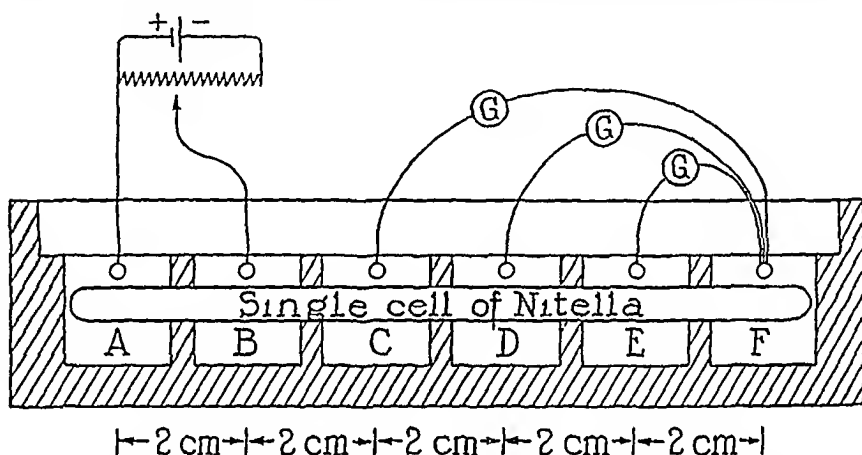
Fɪɢ 3 Diagram of a series of paraffin cups A to F, with a single cell of *Nitella* passing through all of them   (In each partition the *Nitella* cell is sealed in with vaseline) GGG as in Fig 1   Ag-AgCl electrodes dip into the cups   *Cf* Oster-hout, W J V , and Hill, S E , *J Gen Physiol* , 1933–34, **17**, 87

lot of cells which had lost their irritability and potassium effect were soaked in 0 001 ᴍ or 0 002 ᴍ NH₃   An electrical stimulus giving an outgoing current at B was then applied (100 to 300 mv ᴅ ᴄ) and they were then tested for the potassium effect

In the first set of experiments it was found that soaking for 1 minute sufficed to restore the potassium effect but not the irritability   In order to ascertain the proper length of exposure the following procedure was adopted

[9] Restoration of the potassium effect was not regarded as due to NH₃ when preceded by an action current since the latter can itself cause an action current (footnote 2)

Cells were arranged as in Fig 3 with 0 01 M NaCl in all the cups   Then the NaCl was replaced by a solution of NH₃ which was poured in until it filled the cups and covered the spaces between them so that it bathed the entire cell   After 15 seconds it was drained off so that it filled the cups but left the spaces between them free from solution   in these spaces the cell was surrounded by moist air (the cell wall was imbibed with the solution of NH₃)   The cell was then tested for potassium effect and irritability and then completely submerged once more in the solution of NH₃ for 15 seconds   This was continued until a positive result was obtained [9]

Using this procedure it was found that an exposure (varying from 45 seconds to 2 minutes) to 0 0005 M or 0 001 M NH₃ or to 0 01 M NH₄Cl (at pH about 5 4) sufficed to restore the potassium effect and irritability in nearly every cell tested

The action curves were like those in normal cells except that recovery was often slow   in many cases they belonged to types which, though occurring in normal cells, were not those most commonly encountered in such cells

DISCUSSION

How does NH₃ produce these effects?   Alkalinity does not seem to be of primary importance since similar effects are obtained with NH₄Cl 0 01 M at a pH of about 5 4 (where the concentration of NH₃ is negligible)   Moreover a number of bases were found to have no restorative action, e g strychnine, brucine, codeine, veratrine, yohimbine, aniline, and toluidine [10]

Previous experiments indicate that the potassium effect depends chiefly on the higher mobility[11] or higher concentration of K^+ in the outer protoplasmic surface (as compared with Na^+)   Such a high mobility of K^+ does not accord with Walden's rule[1] which states that mobility times viscosity is a constant   On this basis the mobility ratio $U_K - U_{Na}$ would be approximately the same in all solvents But great differences in mobility might result from the formation of

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[10] Osterhout, W J V, and Hill S E, *Proc Soc Exp Biol and Med*, 1934–35, 32, 715   The alkaloids were dissolved in water without addition of alkali

[11] Osterhout W J V, *J Gen Physiol*, 1929–30, 13, 715, *Ergebn Physiol*, 1933, 35, 994

[12] Osterhout, W J V, *Ergebn Physiol*, 1933 35, 1007   This rule applies best to large ions

complex ions [13]   If, for example, sodium formed a complex ion of low mobility but potassium did not the situation in normal cells might be accounted for and it would then be necessary to suppose that after treatment with distilled water this no longer happened and in consequence the mobility of Na became approximately equal to that of K^-.   But such an explanation fails where we find that the mobility of Na^- is not changed by the treatment with distilled water since the concentration effect[14] of Na^+ remains the same but that of K^+ is reduced and becomes like that of Na^+

It will be noted that in Fig 2 the substitution of 0 01 M KCl for 0 01 M NaCl causes the P D to become more positive   This would indicate that the apparent mobility of K^+ has become less than that of Na^-

In calculating the mobility of K^+ we use the equation[1]

$$\mathrm{P\,D} = \frac{RT}{F}\frac{u - v}{u + v} \log \frac{C_1}{C_2}$$

so that the result depends in part on the concentration of K^+ in the non-aqueous protoplasmic surface   Could this be greater than the corresponding concentration of Na^+?

It does not seem probable that compounds of potassium in the external solution can have partition coefficients sufficiently high (as compared with those of sodium compounds) to account for the results [15]

But it is possible that potassium may form in the non-aqueous layer compounds yielding K^- or complex ions of the type $(KZ)^+$ and that this may take place to a much greater degree with potassium than with sodium   If $(KZ)^-$ were formed we should, in determining the concentration effect, measure a diffusion potential involving both K^+ and $(KZ)^-$ which would give a higher value for potassium than for sodium

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[13] Solvation with the non-aqueous organic solvent forming the surface layer might play a rôle but it does not seem probable that it could explain the difference in behavior of K^+ and Na^+

[14] $E\,g$ the P D between 0 01 M and 0 001 M NaCl in the protoplasmic surface Cf footnote 1

[15] Shedlovsky, T, and Uhlig, H H   J Gen Physiol, 1933–34, 17, 549, 563

If this did not happen in cells treated with distilled water[16] the observed result might be accounted for   Such compounds would presumably have very little solubility in water [17]

How ammonia alters these, or other variables, to restore the potassium effect cannot be stated at present   Experiments are being made to ascertain what other substances act in similar fashion [19]

One way of attacking this problem is to ascertain whether ammonia affects the behavior of K^+ and of Na^+ alike   For this purpose the concentration effects were determined [18]   It was found that on replacing 0 001 M by 0 01 M NaCl in cells leached with distilled water the concentration effect was about the same as in normal cells   But the concentration effect of KCl was reduced to approximately that of NaCl

Hence it is apparent that leaching affects the behavior of potassium very much more than that of sodium   It may conceivably prevent potassium from combining with substances in the surface layer and hence may decrease the concentration of K^+ or $(KZ)^+$ as compared with that of Na^+ Or it may alter the mobility of K^+

It may be added that when the potassium effect is rapidly restored (within 10 seconds or less), as may happen when 0 001 M NH_3 + 0 01 M KCl is placed on a spot which previously showed no potassium effect with KCl alone, its action must be largely confined to the outer protoplasmic surface layer   For in this brief interval it must pass through the cellulose wall (about 10 microns thick) and it does not have time to penetrate the protoplasm to any great extent

Let us now consider irritability   Previous experiments indicate[19] that unless the non aqueous surface is broken down by the applied E M F , or becomes more permeable, no action current results   If the surface layer is so changed by the leaching that it cannot so readily break down or become permeable, irritability will be correspondingly

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[16] I e   because certain substances had been removed from the surface layer

[17] Compounds of sodium and potassium less soluble in water than in some non polar solvents have been described by Hundeshagen (cf Jacques, A G , J Gen Physiol , 1934–35, 18, 283)

[18] The technique was as described in a former paper (cf footnote 1)

[19] Osterhout, W J V , J Gen Physiol , 1934–35, 18, 215   Hill, S E , and Osterhout, W J V , J Gen Physiol , 1934–35, 18, 377

diminished   On this basis the restorative action of ammonia would depend on its ability to restore the sensitivity of the layers, particularly of that at the inner surface which seems to be the one chiefly concerned [20]

On this basis we should expect the potassium effect to be restored more rapidly than irritability by NH_3 since the potassium effect depends on the condition of the outer surface   This agrees with observation   (But in experiments with NH_4Cl this was not the case the reason for this is not clear)   We should likewise expect the potassium effect to disappear before irritability when cells are placed in distilled water   This often happens[2] but in some cases irritability disappears first

On the basis of what has been said we should expect irritability and the potassium effect to be restored by any substance which can put the surfaces into a condition similar to that found in normal cells in winter[21] and it seems possible that this might be done by a variety of substances   We need not suppose that all cells which show irritability and the potassium effect have precisely the same composition and indeed the notion of uniformity in this respect is opposed by a variety of evidence [22]

We must also bear in mind the possibility that NH_3, NH_4^+, and tetraethyl ammonium cause R to pass from the vacuole into the protoplasm and thus restore the irritability and the potassium effect

[20] Osterhout, W J V , *J Gen Physiol* , 1934–35, 18, 215   It should be borne in mind that the inner protoplasmic surface layer does not appear to change its behavior toward potassium since the P D across the protoplasm does not diminish as the result of treatment with distilled water (this P D appears to be due to the gradient of K^+ across the inner protoplasmic surface)

[21] In summer the cells as a rule do not produce action currents when stimulated electrically

[22] For example, the potassium effect is not always the same in magnitude or in the time required to reach a steady value, indicating that the outer surface is variable   Cells which are irritable may show different forms of the first part of the action curve, indicating that the inner surface is variable (Hill, S E , and Osterhout, W J V , *J Gen Physiol* , 1934–35, 18, 377   Osterhout, W J V , and Hill, S E , *J Gen Physiol* , 1934–35, 18, 499)

SUMMARY

The potassium effect in *Nitella* (the high P D observed in leading off from a spot in contact with 0 01 M KCl to one in contact with 0 01 M NaCl) and the irritability can be removed by placing cells in distilled water for 2 or 3 days   They can be restored by NH_3 or by NH_4Cl The potassium effect can also be restored by tetraethyl ammonium chloride (no tests were made of its ability to restore irritability)

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